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(71) Applicant *(for all designated States except US)*: QUARK BIOTECH, INC. [US/US]; 1059 Serpentine Lane, Pleasanton, CA 94566 (US).

(71) Applicant *(for MW only)*: KOHN, Kenneth [US/US]; 6761 Alderley Way, West Bloomfield, MI 48322 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: EINAT, Paz [IL/IL]; Neve Nir 1/27, 74042 Nes-Ziona (IL). SKALITER, Rami [IL/IL]; Habanim 117/10, 74037 Nes-Ziona (IL). FEINSTEIN, Elena [IL/IL]; Hahagana Street 12/29, 76214 Rehovot (IL).

(74) Agent: KOHN, Kenneth; Kohn & Associates, Suite 410, 30500 Northwestern Highway, Farmington Hills, MI 48334 (US).

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(54) Title: SEQUENCES CHARACTERISTIC OF HYPOXIA-REGULATED GENE TRANSCRIPTION

(57) Abstract

There are provided polynucleotide sequences for use in identifying genes which are modulated by hypoxic conditions.

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SEQUENCES CHARACTERISTIC OF HYPOXIA-REGULATED GENE TRANSCRIPTION

BACKGROUND OF THE INVENTION

TECHNICAL FIELD

The present invention relates to the identification of genes that are differentially expressed in hypoxia and use of the genes and gene products for diagnosis and therapeutic intervention. The invention further relates to identification of polynucleotide sequences that are differentially expressed in hypoxia and the use of the sequences for diagnosis and probes.

BACKGROUND ART

The level of tissue oxygenation plays an important role in normal development as well as in pathologic processes such as ischemia. Tissue oxygenation plays a significant regulatory/inducer role in both apoptosis and in angiogenesis (Bouck et al, 1996; Bunn et al, 1996; Dor et al, 1997; Carmeliet et al, 1998). Apoptosis (see Duke et al, 1996 for review) and growth arrest occur when cell growth and viability are reduced due to oxygen deprivation (hypoxia). Angiogenesis (i.e. blood vessel growth, vascularization) is stimulated when hypo-oxygenated cells secrete factors which stimulate proliferation and migration of endothelial cells in an attempt to restore oxygen homeostasis (for review see Hanahan et al, 1996).

Hypoxia plays a critical role in the selection of mutations that contribute to more severe tumorogenic phenotypes (Graeber et al., 1996). Identifying activated or inactivated genes and gene products in hypoxia and ischemia is needed.

Ischemic disease pathologies involve a decrease in the blood supply to a bodily organ, tissue or body part generally caused by constriction or obstruction of the blood vessels, as for example retinopathy, myocardial infarction and stroke. Therefore, apoptosis and/or angiogenesis as induced by the ischemic condition are also involved in these disease states. Neoangiogenesis is seen in some forms of retinopathy and in tumor growth. These processes are complex cascades of events controlled by many different genes reacting to the various stresses such as hypoxia.

The ability to monitor hypoxia-triggered activation of genes can provide a tool to identify not immediately evident ischemia in a patient. Identification of hypoxia-regulated genes permits the utilization of gene therapy or direct use of gene products, or alternatively inactivation of target genes for therapeutic intervention in treating the diseases and pathologies associated with hypoxia, ischemia and tumor growth.

SUMMARY OF THE INVENTION

The present invention provides purified, isolated and cloned polynucleotides (nucleic acid sequences) associated with hypoxia-regulated activity and having sequences designated as any one of SEQ ID NOS. 1-9, or having complementary or allelic variation sequences thereto.

The present invention provides a method of regulating angiogenesis or apoptosis in a patient in need of such treatment by administering to such patient a therapeutically effective amount of an antagonist of at least one protein as encoded by the nucleic acid sequences as set forth in any of SEQ ID NOS. 1-9.

Also provided is the diagnostic tool for identifying genes modulated by hypoxic conditions having a detector for detecting the presence of a polynucleotide having a nucleic acid sequence according to any of SEQ. ID NOS. 1-9. A pharmaceutical composition for modulating hypoxia and ischemia having an effective amount of a polynucleotide having the nucleic acid sequence according to SEQ. ID NOS. 1-9 and a pharmaceutically acceptable carrier is also provided.

Also provided is a method of regulating hypoxia-associated pathologies by administering an effective amount of at least one antisense oligonucleotide against one of the nucleic acid sequences (SEQ. ID NOS. 1-9) or their proteins. There is provided a method of regulating hypoxia associated pathology by administering an effective amount of a protein encoded by the polynucleotides (SEQ. ID NOS. 1-9) as active ingredients in the pharmaceutically acceptable carrier.

Further, there are provided hypoxia response regulating genes.

BRIEF DESCRIPTION OF THE FIGURES

Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

Figure 1 shows the nucleic acid sequence of the 92 gene (Seq. I.D. No. 1);

Figure 2 shows the nucleic acid sequence of the 95 gene (Seq. I.D. No. 2);

Figure 3 shows the nucleic acid sequence of the 98 gene (Seq. I.D. No. 3);

Figure 4 shows the nucleic acid sequence of the 60F6 gene (Seq. I.D. No. 4);

Figures 5 A-C show the nucleic acid sequence of the 648 gene (Seq. I.D. No. 5);

Figure 6 shows the nucleic acid sequence of the 24D4 gene (Seq. I.D. No. 6);

Figures 7 A and B show the nucleic acid sequence of the 77H4 gene (Seq. I.D. No. 7);

Figure 8 shows the nucleic acid sequence of the 14G2 gene (Seq. I.D. No. 8); and

Figure 9 shows the nucleic acid sequence of the 29F3 gene (Seq. I.D. No. 9).

DESCRIPTION OF THE INVENTION

The present invention identifies polynucleotides (nucleic acid sequences) with sequences as set forth herein in SEQ. ID Nos. 1-9, that can be utilized diagnostically in hypoxia and ischemia and that can be used as targets for therapeutic intervention, or can be used to identify genes that are regulated and respond to hypoxic conditions. SEQ ID Nos. 1-4 and 6-8 have not previously been identified. SEQ ID No. 5 was found to match sequences in data banks but has not been reported to be associated with hypoxia regulation.

The present invention further provides candidate genes and gene products that can be utilized therapeutically and diagnostically in hypoxia and ischemia and that can regulate apoptosis or angiogenesis.

By regulate or modulate or control, it is meant that the process is either induced or inhibited to the degree necessary to effect a change in the process and the associated disease state in the patient. Whether induction or inhibition is being contemplated will be apparent from the process and disease being treated and will be known to those skilled in the medical arts.

The present invention identifies genes for gene therapy, diagnostics and therapeutics that have direct causal relationships between a disease and its related pathologies and up- or down-regulator (responder) genes. That is, the present invention is initiated by a physiological relationship between cause and effect.

The present invention also provides a method of regulating angiogenesis or apoptosis in a patient in need of such treatment by administering to such patient a therapeutically effective amount of an antagonist of at least one protein as encoded by the nucleic acid sequences as set forth in any of SEQ ID NOS. 1-9.

As shown below, some sequences are partial gene sequences which are markers/probes for genes that are upregulated under hypoxic conditions. These partial sequences can be designated "Expressed Sequence Tags" (ESTs) and are markers for the genes actually expressed *in vivo* and are ascertained as described herein in the Examples or as is known in the art.

Generally, ESTs comprise DNA sequences corresponding to a portion of nuclear encoded mRNA. The EST has a length that allows for PCR (polymerase chain reaction), for use as a hybridization probe and is a

unique designation for the gene with which it hybridizes (generally under conditions sufficiently stringent to require at least 95% base pairing). For a detailed description and review of ESTs and their functional utility see, WO 93/00353 PCT Application which is incorporated herein in its entirety by reference, as well as the references by Zweiger et al, 1997; Okubo et al, 1997 and Braren et al, 1997.

The WO 93/00353 PCT application further describes how the EST sequences can be used to identify the transcribed genes.

The present invention also provides a method of diagnosing the presence of ischemia or other hypoxia-associated pathologies in a patient including the steps of analyzing a tissue sample from the patient for the presence of at least one expressed gene (up-regulated) identified by the sequences of the present invention utilized as probes. Methods of identification of hybridization can include immunohistochemical staining of the tissue samples. Further, for identification of the gene, *in situ* hybridization, Southern blotting, single strand conformational polymorphism, restriction endonuclease fingerprinting (REF), PCR amplification and DNA-chip analysis using the nucleic acid sequences of the present invention as probes/primers can be used.

Further, according to the present invention, purified, isolated and cloned hypoxia-responding genes identified by the probes/sequences hybridizing under stringent conditions with 95% homology set forth herein or a complementary or allelic variation sequence and human homologies as needed thereto are disclosed. The present invention further provides proteins as encoded by the identified genes. The present invention further provides antibodies directed against these proteins. The present

invention further provides transgenic animals and cell lines carrying at least one expressible gene identified by the present invention. The present invention further provides knock-out eucaryotic organisms in which at least one nucleic acid sequences as identified by the probes of the present invention.

The present invention provides a method of regulating angiogenesis, apoptosis or other hypoxia-associated pathologies in a patient in need of such treatment by administering to a patient a therapeutically effective amount of an antagonist of at least one protein as encoded by the nucleic acid sequences or sequences identified herein or by the probes of the present invention, or alternatively by a non-protein product of the gene's activity, or inactivation of a gene by chemical compound. Alternatively, the present invention provides a method of regulating angiogenesis, apoptosis or other hypoxia-associated pathologies in a patient in need of such treatment by administering to a patient a therapeutically effective amount of at least one antisense oligonucleotide against the nucleic acid sequences or dominant negative peptide directed against the sequences or their proteins.

The present invention further provides a method of regulating angiogenesis or apoptosis in a patient in need of such treatment by administering to a patient a therapeutically effective amount of a protein encoded by the identified genes as active ingredients in a pharmaceutically acceptable carrier.

The present invention provides a method of providing an apoptosis-regulating gene, an angiogenesis-regulating gene or hypoxia-response regulating gene identified by the probes of the present invention, by administering directly to a patient in need of such therapy an expressible vector comprising expression

control sequences operably linked to one of the identified genes and its human homolog if appropriate.

The proteins of the present invention can be produced recombinantly (see generally Marshak et al, 1996 "Strategies for Protein Purification and Characterization. A laboratory course manual." CSHL Press) and analogues can be due to post-translational processing. The term Analogue as used herein is defined as a nucleic acid sequence or protein which has some differences in their amino acid/nucleotide sequences as compared to the native sequence of SEQ. ID NOS. 1-9. Ordinarily, the analogue will be generally at least 70% homologous over any portion that is functionally relevant. In more preferred embodiments the homology will be at least 80% and can approach 95% homology to the protein/nucleotide sequence.

The amino acid or nucleotide sequence of an analogue can differ from that of the primary sequence when at least one residue is deleted, inserted or substituted, but the protein or nucleic acid molecule remains functional. Differences in glycosylation can provide protein analogues.

Functionally relevant refers to the biological property of the molecule and in this context means an *in vivo* effector or antigenic function or activity that is directly or indirectly performed by a naturally occurring protein or nucleic acid molecule. Effector functions include but are not limited to include receptor binding, any enzymatic activity or enzyme modulatory activity, any carrier binding activity, any hormonal activity, any activity in promoting or inhibiting adhesion of cells to extracellular matrix or cell surface molecules, or any structural role as well as having the nucleic acid sequence encode functional protein and be expressible. The antigenic functions essentially mean the possession of an

epitope or antigenic site that is capable of cross-reacting with antibodies raised against a naturally occurring protein. Biologically active analogues share an effector function of the native which can, but need not, in addition possess an antigenic function.

The antibodies can be either monoclonal, polyclonal or recombinant and be used in immunoassays. Conveniently, the antibodies can be prepared against the immunogen or portion thereof for example a synthetic peptide based on the sequence, or prepared recombinantly by cloning techniques or the natural gene product and/or portions thereof can be isolated and used as the immunogen. Immunogens can be used to produce antibodies by standard antibody production technology well known to those skilled in the art as described generally in Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988 and Borrebaeck, *Antibody Engineering - A Practical Guide*, W.H. Freeman and Co., 1992. Antibody fragments can also be prepared from the antibodies and include Fab, $F(ab')_2$, and Fv by methods known to those skilled in the art.

For producing polyclonal antibodies a host, such as a rabbit or goat, is immunized with the immunogen or immunogen fragment, generally with an adjuvant and, if necessary, coupled to a carrier; antibodies to the immunogen are collected from the sera. Further, the polyclonal antibody can be absorbed such that it is monospecific. That is, the sera can be absorbed against related immunogens so that no cross-reactive antibodies remain in the sera rendering it monospecific.

For producing monoclonal antibodies the technique involves hyperimmunization of an appropriate donor with the immunogen, generally a mouse, and isolation of splenic antibody producing cells. These cells are

fused to a cell having immortality, such as a myloma cell, to provide a fused cell hybrid which has immortality and secretes the required antibody. The cells are then cultured, in bulk, and the monoclonal antibodies harvested from the culture media for use.

For producing recombinant antibody (see generally Huston et al, 1991; Johnson and Bird, 1991; Mernaugh and Mernaugh, 1995), messenger RNAs from antibody producing B-lymphocytes of animals, or hybridoma are reverse-transcribed to obtain complimentary DNAs (cDNAs). Antibody cDNA, which can be full or partial length, is amplified and cloned into a phage or a plasmid. The cDNA can be a partial length of heavy and light chain cDNA, separated or connected by a linker. The antibody, or antibody fragment, is expressed using a suitable expression system to obtain recombinant antibody. Antibody cDNA can also be obtained by screening pertinent expression libraries.

The antibody can be bound to a solid support substrate or conjugated with a detectable moiety or be both bound and conjugated, as is well known in the art. (For a general discussion of conjugation of fluorescent or enzymatic moieties see Johnstone & Thorpe, *Immunochemistry in Practice*, Blackwell Scientific Publications, Oxford, 1982.) The binding of antibodies to a solid support substrate is also well known in the art. (See for a general discussion Harlow & Lane *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Publications, New York, 1988 and Borrebaeck, *Antibody Engineering - A Practical Guide*, W.H. Freeman and Co., 1992). The detectable moieties contemplated with the present invention can include, but are not limited to, fluorescent, metallic, enzymatic and radioactive markers such as biotin, gold, ferritin, alkaline phosphatase, β-

galactosidase, peroxidase, urease, fluorescein, rhodamine, tritium, ^{14}C and iodination.

These transgenics and knock-outs of the present invention are constructed using standard methods known in the art and as set forth in United States Patents 5,487,992, 5,464,764, 5,387,742, 5,360,735, 5,347,075, 5,298,422, 5,288,846, 5,221,778, 5,175,385, 5,175,384, 5,175,383, 4,736,866 as well as Burke and Olson (1991), Capecchi (1989), Davies et al. (1992), Dickinson et al. (1993), Duff and Lincoln (1995), Huxley et al. (1991), Jakobovits et al. (1993), Lamb et al. (1993), Pearson and Choi (1993), Rothstein (1991), Schedl et al. (1993), Strauss et al. (1993). Further, patent applications WO 94/23049, WO 93/14200, WO 94/06908, WO 94/28123 also provide information.

More specifically, any techniques known in the art can be used to introduce the transgene expressibly into animals to produce the parental lines of animals. Such techniques include, but are not limited to, pronuclear microinjection (U.S. patent 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten et al., 1985); gene targeting in embryonic stem cells (Thompson et al., 1989; Mansour, 1990 and U.S. patent 5,614,396); electroporation of embryos (Lo, 1983); and sperm-mediated gene transfer (Lavitrano et al., 1989). For a review of such techniques see Gordon (1989).

Further, one parent strain instead of carrying a direct human transgene can have the homologous endogenous gene modified by gene targeting such that it approximates the transgene. That is, the endogenous gene has been "humanized" and/or mutated (Reaume et al, 1996). It should be noted that if the animal and human sequence are essentially homologous a "humanized" gene is not required. The transgenic parent can also carry an over expressed sequence, either the non-mutant or a mutant sequence and

humanized or not as required. The term transgene is therefore used to refer to all these possibilities.

Additionally, cells can be isolated from the offspring which carry a transgene from each transgenic parent and that are used to establish primary cell cultures or cell lines as is known in the art.

Where appropriate, a parent strain will be homozygous for the transgene. Additionally, where appropriate, the endogenous non-transgene in the genome that is homologous to the transgene will be non-expressive. By non-expressive is meant that the endogenous gene will not be expressed and that this non-expression is heritable in the offspring. For example, the endogenous homologous gene could be "knocked-out" by methods known in the art. Alternatively, the parental strain that receives one of the transgenes could carry a mutation at the endogenous homologous gene rendering it non-expressed.

The antagonist/regulating agent/active ingredient is dosed and delivered in a pharmaceutically acceptable carrier as described herein below. The term antagonist or antagonizing is used in its broadest sense. Antagonism can include any mechanism or treatment which results in inhibition, inactivation, blocking or reduction in gene activity or gene product. It should be noted that the inhibition of a gene or gene product can provide for an increase in a corresponding function that the gene or gene product was regulating. The antagonizing step can include blocking cellular receptors for the gene products and can include antisense treatment as discussed herein below. For example, a patient can be in need of inducing apoptosis in tumorogenic cells or angiogenesis in trauma situations where for example a limb must be reattached or in a transplant where revascularization is needed.

Many reviews have covered the main aspects of

antisense (AS) technology and its enormous therapeutic potential (Wright and Anazodo, 1995). There are reviews on the chemical (Crooke, 1995; Uhlmann et al, 1990), cellular (Wagner, 1994) and therapeutic (Hanania, *et al*, 1995; Scanlon, *et al*, 1995; Gewirtz, 1993) aspects of this rapidly developing technology. Within a relatively short time, ample information has accumulated about the *in vitro* use of AS nucleotide sequences in cultured primary cells and cell lines as well as for *in vivo* administration of such nucleotide sequences for suppressing specific processes and changing body functions in a transient manner. Further, enough experience is now available *in vitro* and *in vivo* in animal models and human clinical trials to predict human efficacy.

Antisense intervention in the expression of specific genes can be achieved by the use of synthetic AS oligonucleotide sequences (for recent reports see Lefebvre-d'Hellencourt *et al*, 1995; Agrawal, 1996; Lev-Lehman *et al*, 1997). AS oligonucleotide sequences can be short sequences of DNA, typically 15-30 mer but can be as small as 7 mer (Wagner et al, 1996), designed to complement a target mRNA of interest and form an RNA:AS duplex. This duplex formation can prevent processing, splicing, transport or translation of the relevant mRNA. Moreover, certain AS nucleotide sequences can elicit cellular RNase H activity when hybridized with their target mRNA, resulting in mRNA degradation (Calabretta *et al*, 1996). In that case, RNase H will cleave the RNA component of the duplex and can potentially release the AS to further hybridize with additional molecules of the target RNA. An additional mode of action results from the interaction of AS with genomic DNA to form a triple helix which can be transcriptionally inactive.

The sequence target segment for the antisense oligonucleotide is selected such that the sequence exhibits suitable energy related characteristics important for oligonucleotide duplex formation with their complementary templates, and shows a low potential for self-dimerization or self-complementation [Anazodo et al., 1996]. For example, the computer program OLIGO (Primer Analysis Software, Version 3.4), can be used to determine antisense sequence melting temperature, free energy properties, and to estimate potential self-dimer formation and self-complimentary properties. The program allows the determination of a qualitative estimation of these two parameters (potential self-dimer formation and self-complimentary) and provides an indication of "no potential" or "some potential" or "essentially complete potential". Using this program target segments are generally selected that have estimates of no potential in these parameters. However, segments can be used that have "some potential" in one of the categories. A balance of the parameters is used in the selection as is known in the art. Further, the oligonucleotides are also selected as needed so that analogue substitution do not substantially affect function.

Phosphorothioate antisense oligonucleotides do not normally show significant toxicity at concentrations that are effective and exhibit sufficient pharmacodynamic half-lives in animals (Agarwal *et al.*, 1996) and are nuclease resistant. Antisense induced loss-of-function phenotypes related with cellular development were shown for the glial fibrillary acidic protein (GFAP), for the establishment of tectal plate formation in chick (Galileo *et al.*, 1991) and for the N-myc protein, responsible for the maintenance of cellular heterogeneity in neuroectodermal cultures (epithelial vs. neuroblastic

cells, which differ in their colony forming abilities, tumorogenicity and adherence) (Rosolen *et al.*, 1990; Whitesell *et al*, 1991). Antisense oligonucleotide inhibition of basic fibroblast growth factor (bFgF), having mitogenic and angiogenic properties, suppressed 80% of growth in glioma cells (Morrison, 1991) in a saturable and specific manner. Being hydrophobic, antisense oligonucleotides interact well with phospholipid membranes (Akhter *et al.*, 1991). Following their interaction with the cellular plasma membrane, they are actively (or passively) transported into living cells (Loke *et al.*, 1989), in a saturable mechanism predicted to involve specific receptors (Yakubov *et al.*, 1989).

Instead of an antisense sequence as discussed herein above, ribozymes can be utilized. This is particularly necessary in cases where antisense therapy is limited by stoichiometric considerations (Sarver et al., 1990, Gene Regulation and Aids, pp. 305-325). Ribozymes can then be used that will target the same sequence. Ribozymes are RNA molecules that possess RNA catalytic ability (see Cech for review) that cleave a specific site in a target RNA. The number of RNA molecules that are cleaved by a ribozyme is greater than the number predicted by stochiochemistry. (Hampel and Tritz, 1989; Uhlenbeck, 1987).

Ribozymes catalyze the phosphodiester bond cleavage of RNA. Several ribozyme structural families have been identified including Group I introns, RNase P, the hepatitis delta virus ribozyme, hammerhead ribozymes and the hairpin ribozyme originally derived from the negative strand of the tobacco ringspot virus satellite RNA (sTRSV) (Sullivan, 1994; U.S. Patent No. 5,225,347, columns 4-5). The latter two families are derived from viroids and virusoids, in which the ribozyme is believed

to separate monomers from oligomers created during rolling circle replication (Symons, 1989 and 1992). Hammerhead and hairpin ribozyme motifs are most commonly adapted for trans-cleavage of mRNAs for gene therapy (Sullivan, 1994). The ribozyme type utilized in the present invention is selected as is known in the art. Hairpin ribozymes are now in clinical trial and are the preferred type. In general the ribozyme is from 30-100 nucleotides in length.

Modifications or analogues of nucleotides can be introduced to improve the therapeutic properties of the nucleotides. Improved properties include increased nuclease resistance and/or increased ability to permeate cell membranes.

Nuclease resistance, where needed, is provided by any method known in the art that does not interfere with biological activity of the antisense oligodeoxynucleotides, cDNA and/or ribozymes as needed for the method of use and delivery (Iyer et al., 1990; Eckstein, 1985; Spitzer and Eckstein, 1988; Woolf et al., 1990; Shaw et al., 1991). Modifications that can be made to oligonucleotides in order to enhance nuclease resistance include modifying the phosphorous or oxygen heteroatom in the phosphate backbone. These include preparing methyl phosphonates, phosphorothioates, phosphorodithioates and morpholino oligomers. In one embodiment it is provided by having phosphorothioate bonds linking between the four to six 3'-terminus nucleotide bases. Alternatively, phosphorothioate bonds link all the nucleotide bases. Other modifications known in the art can be used where the biological activity is retained, but the stability to nucleases is substantially increased.

The present invention also includes all analogues of, or modifications to, an oligonucleotide of the invention that does not substantially affect the function of the oligonucleotide. The nucleotides can be

selected from naturally occurring or synthetic modified bases. Naturally occurring bases include adenine, guanine, cytosine, thymine and uracil. Modified bases of the oligonucleotides include xanthine, hypoxanthine, 2-aminoadenine, 6-methyl, 2-propyl and other alkyl adenines, 5-halo uracil, 5-halo cytosine, 6-aza cytosine and 6-aza thymine, pseudo uracil, 4-thiuracil, 8-halo adenine, 8-aminoadenine, 8-thiol adenine, 8-thiolalkyl adenines, 8-hydroxyl adenine and other 8-substituted adenines, 8-halo guanines, 8-amino guanine, 8-thiol guanine, 8-thioalkyl guanines, 8-hydroxyl guanine and other substituted guanines, other aza and deaza adenines, other aza and deaza guanines, 5-trifluoromethyl uracil and 5-trifluoro cytosine.

In addition, analogues of nucleotides can be prepared wherein the structure of the nucleotide is fundamentally altered and that are better suited as therapeutic or experimental reagents. An example of a nucleotide analogue is a peptide nucleic acid (PNA) wherein the deoxyribose (or ribose) phosphate backbone in DNA (or RNA0 is replaced with a polyamide backbone which is similar to that found in peptides. PNA analogues have been shown to be resistant to degradation by enzymes and to have extended lives *in vivo* and *in vitro*. Further, PNAs have been shown to bind stronger to a complementary DNA sequence than a DNA molecule. This observation is attributed to the lack of charge repulsion between the PNA strand and the DNA strand. Other modifications that can be made to oligonucleotides include polymer backbones, cyclic backbones, or acyclic backbones.

The active ingredients of the pharmaceutical composition can include oligonucleotides that are nuclease resistant needed for the practice of the invention or a fragment thereof shown to have the same effect targeted

against the appropriate sequence(s) and/or ribozymes. Combinations of active ingredients as disclosed in the present invention can be used including combinations of antisense sequences.

The antisense oligonucleotides (and/or ribozymes) and cDNA of the present invention can be synthesized by any method known in the art for ribonucleic or deoxyribonucleic nucleotides. For example, an Applied Biosystems 380B DNA synthesizer can be used. When fragments are used, two or more such sequences can be synthesized and linked together for use in the present invention.

The nucleotide sequences of the present invention can be delivered either directly or with viral or non-viral vectors. When delivered directly the sequences are generally rendered nuclease resistant. Alternatively, the sequences can be incorporated into expression cassettes or constructs such that the sequence is expressed in the cell as discussed herein below. Generally, the construct contains the proper regulatory sequence or promoter to allow the sequence to be expressed in the targeted cell.

Negative dominant peptide refers to a partial cDNA sequence that encodes for a part of a protein, i.e. a peptide (see Herskowitz, 1987). This peptide can have a different function from the protein it was derived from. It can interact with the full protein and inhibit its activity or it can interact with other proteins and inhibit their activity in response to the full protein. Negative dominant means that the peptide is able to overcome the natural proteins and fully inhibit their activity to give the cell a different characteristics like resistance or sensitization to killing. For therapeutic intervention either the peptide itself is delivered as the active ingredient of a pharmaceutical composition or the

cDNA can be delivered to the cell utilizing the same methods as for antisense delivery.

By gene therapy as used herein refers to the transfer of genetic material (e.g. DNA or RNA) of interest into a host to treat or prevent a genetic or acquired disease or condition phenotype. The genetic material of interest encodes a product (e.g. a protein, polypeptide, peptide, functional RNA, antisense) whose production *in vivo* is desired. For example, the genetic material of interest can encode a hormone, receptor, enzyme, polypeptide or peptide of therapeutic value. Alternatively, the genetic material of interest encodes a suicide gene. For a review see, in general, the text "Gene Therapy" (Advances in Pharmacology 40, Academic Press, 1997).

Two basic approaches to gene therapy have evolved: (1) *ex vivo* and (2) *in vivo* gene therapy. In *ex vivo* gene therapy cells are removed from a patient, and while being cultured are treated *in vitro*. Generally, a functional replacement gene is introduced into the cell via an appropriate gene delivery vehicle/method (transfection, transduction, homologous recombination, etc.) and an expression system as needed and then the modified cells are expanded in culture and returned to the host/patient. These genetically reimplanted cells have been shown to express the transfected genetic material *in situ*.

In *in vivo* gene therapy, target cells are not removed from the subject rather the genetic material to be transferred is introduced into the cells of the recipient organism *in situ*, that is within the recipient. In an alternative embodiment, if the host gene is defective, the gene is repaired *in situ* [Culver, 1998]. These

genetically altered cells have been shown to express the transfected genetic material *in situ*.

The gene expression vehicle is capable of delivery/transfer of heterologous nucleic acid into a host cell. The expression vehicle can include elements to control targeting, expression and transcription of the nucleic acid in a cell selective manner as is known in the art. It should be noted that often the 5' UTR and/or 3' UTR of the gene can be replaced by the 5' UTR and/or 3' UTR of the expression vehicle. Therefore as used herein the expression vehicle can, as needed, not include the 5' UTR and/or 3' UTR of the actual gene to be transferred and only include the specific amino acid coding region.

The expression vehicle can include a promoter for controlling transcription of the heterologous material and can be either a constitutive or inducible promoter to allow selective transcription. Enhancers that can be required to obtain necessary transcription levels can optionally be included. Enhancers are generally any non-translated DNA sequence which works contiguously with the coding sequence (in cis) to change the basal transcription level dictated by the promoter. The expression vehicle can also include a selection gene as described herein below.

Vectors can be introduced into cells or tissues by any one of a variety of known methods within the art. Such methods can be found generally described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1989, 1992), in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989), Chang et al., *Somatic Gene Therapy*, CRC Press, Ann Arbor, MI (1995), Vega et al., *Gene Targeting*, CRC Press, Ann Arbor, MI (1995), *Vectors: A Survey of Molecular Cloning Vectors*

and Their Uses, Butterworths, Boston MA (1988) and Gilboa et al (1986) and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. In addition, see United States patent 4,866,042 for vectors involving the central nervous system and also United States patents 5,464,764 and 5,487,992 for positive-negative selection methods.

Introduction of nucleic acids by infection offers several advantages over the other listed methods. Higher efficiency can be obtained due to their infectious nature. Moreover, viruses are very specialized and typically infect and propagate in specific cell types. Thus, their natural specificity can be used to target the vectors to specific cell types *in vivo* or within a tissue or mixed culture of cells. Viral vectors can also be modified with specific receptors or ligands to alter target specificity through receptor mediated events.

A specific example of DNA viral vector for introducing and expressing recombinant sequences is the adenovirus derived vector Adenop53TK. This vector expresses a herpes virus thymidine kinase (TK) gene for either positive or negative selection and an expression cassette for desired recombinant sequences. This vector can be used to infect cells that have an adenovirus receptor which includes most cancers of epithelial origin as well as others. This vector as well as others that exhibit similar desired functions can be used to treat a mixed population of cells and can include, for example, an *in vitro* or *ex vivo* culture of cells, a tissue or a human subject.

Additional features can be added to the vector to ensure its safety and/or enhance its therapeutic efficacy. Such features include, for example, markers that can be used to negatively select against cells infected

with the recombinant virus. An example of such a negative selection marker is the TK gene described above that confers sensitivity to the antibiotic gancyclovir. Negative selection is therefore a means by which infection can be controlled because it provides inducible suicide through the addition of antibiotic. Such protection ensures that if, for example, mutations arise that produce altered forms of the viral vector or recombinant sequence, cellular transformation will not occur.

Features that limit expression to particular cell types can also be included. Such features include, for example, promoter and regulatory elements that are specific for the desired cell type.

In addition, recombinant viral vectors are useful for *in vivo* expression of a desired nucleic acid because they offer advantages such as lateral infection and targeting specificity. Lateral infection is inherent in the life cycle of, for example, retrovirus and is the process by which a single infected cell produces many progeny virions that bud off and infect neighboring cells. The result is that a large area becomes rapidly infected, most of which was not initially infected by the original viral particles. This is in contrast to vertical-type of infection in which the infectious agent spreads only through daughter progeny. Viral vectors can also be produced that are unable to spread laterally. This characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

As described above, viruses are very specialized infectious agents that have evolved, in many cases, to elude host defense mechanisms. Typically, viruses infect and propagate in specific cell types. The targeting specificity of viral vectors utilizes its natural

specificity to specifically target predetermined cell types and thereby introduce a recombinant gene into the infected cell. The vector to be used in the methods of the invention will depend on desired cell type to be targeted and will be known to those skilled in the art. For example, if breast cancer is to be treated then a vector specific for such epithelial cells would be used. Likewise, if diseases or pathological conditions of the hematopoietic system are to be treated, then a viral vector that is specific for blood cells and their precursors, preferably for the specific type of hematopoietic cell, would be used.

Retroviral vectors can be constructed to function either as infectious particles or to undergo only a single initial round of infection. In the former case, the genome of the virus is modified so that it maintains all the necessary genes, regulatory sequences and packaging signals to synthesize new viral proteins and RNA. Once these molecules are synthesized, the host cell packages the RNA into new viral particles which are capable of undergoing further rounds of infection. The vector's genome is also engineered to encode and express the desired recombinant gene. In the case of non-infectious viral vectors, the vector genome is usually mutated to destroy the viral packaging signal that is required to encapsulate the RNA into viral particles. Without such a signal, any particles that are formed will not contain a genome and therefore cannot proceed through subsequent rounds of infection. The specific type of vector will depend upon the intended application. The actual vectors are also known and readily available within the art or can be constructed by one skilled in the art using well-known methodology.

The recombinant vector can be administered in several ways. If viral vectors are used, for example, the

procedure can take advantage of their target specificity and consequently, do not have to be administered locally at the diseased site. However, local administration can provide a quicker and more effective treatment, administration can also be performed by, for example, intravenous or subcutaneous injection into the subject. Injection of the viral vectors into a spinal fluid can also be used as a mode of administration, especially in the case of neuro-degenerative diseases. Following injection, the viral vectors will circulate until they recognize host cells with the appropriate target specificity for infection.

DNA can also be administered using a gene gun. (Ziao & Brancksman, Nuceic Acids, Res. 24, 2630-2622 (1996)). The DNA is precipitated onto the surface of microscopic metal beads. The microprojectiles are accelerated with a shock wave or expanding helium gas, and penetrate tissues to a depth of several cell layers. For example, the Acal™ Gene Delivery Device manufactured by Aegacetus, Inc., Middleton, WI, is suitable. Alternatively, nucleic DNA can pass through skin into the bloodstream simply by spotting the DNA onto skin with chemical or mechanical irritation (see WO 95/05853).

An alternate mode of administration can be by direct inoculation locally at the site of the disease or pathological condition or by inoculation into the vascular system supplying the site with nutrients or into the spinal fluid. Local administration is advantageous because there is no dilution effect and, therefore, a smaller dose is required to achieve expression in a majority of the targeted cells. Additionally, local inoculation can alleviate the targeting requirement required with other forms of administration since a vector can be used that infects all cells in the inoculated area.

If expression is desired in only a specific subset of cells within the inoculated area, then promoter and regulatory elements that are specific for the desired subset can be used to accomplish this goal. Such non-targeting vectors can be, for example, viral vectors, viral genome, plasmids, phagemids and the like. Transfection vehicles such as liposomes can also be used to introduce the non-viral vectors described above into recipient cells within the inoculated area. Such transfection vehicles are known by one skilled within the art.

The pharmaceutical compositions containing the active ingredients of the present invention as described herein above are administered and dosed in accordance with good medical practice, taking into account the clinical condition of the individual patient, the site and method of administration, scheduling of administration, patient age, sex, body weight and other factors known to medical practitioners. The pharmaceutically "effective amount" for purposes herein is thus determined by such considerations as are known in the medical arts. The amount must be effective to achieve improvement including but not limited to improved survival rate or more rapid recovery, or improvement or elimination of symptoms and other indicators as are selected as appropriate measures by those skilled in the medical arts. The pharmaceutical compositions can be combinations of the active ingredients but will include at least one active ingredient.

In the method of the present invention, the pharmaceutical compositions of the present invention can be administered in various ways taking into account the nature of compounds in the pharmaceutical compositions. It should be noted that they can be administered as the compound or as pharmaceutically acceptable salt and can be administered alone or as an active ingredient in

combination with pharmaceutically acceptable carriers, diluents, adjuvants and vehicles. The compounds can be administered orally, subcutaneously or parenterally including intravenous, intra-arterial, intramuscular, intra-peritoneally, and intra-nasal administration as well as intra-thecal and infusion techniques. Implants of the compounds are also useful. The patient being treated is a warm-blooded animal and, in particular, mammals including man. The pharmaceutically acceptable carriers, diluents, adjuvants and vehicles as well as implant carriers generally refer to inert, non-toxic solid or liquid fillers, diluents or encapsulating material not reacting with the active ingredients of the invention.

It is noted that humans are treated generally longer than the mice or other experimental animals exemplified herein which treatment has a length proportional to the length of the disease process and drug effectiveness. The doses can be single doses or multiple doses over a period of several days, but single doses are preferred.

The doses can be single doses or multiple doses over a period of several days. The treatment generally has a length proportional to the length of the disease process and drug effectiveness and the patient species being treated.

When administering the compound of the present invention parenterally, it will generally be formulated in a unit dosage injectable form (solution, suspension, emulsion). The pharmaceutical formulations suitable for injection include sterile aqueous solutions or dispersions and sterile powders for reconstitution into sterile injectable solutions or dispersions. The carrier can be a solvent or dispersing medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like),

suitable mixtures thereof, and vegetable oils.

Proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Non-aqueous vehicles such a cottonseed oil, sesame oil, olive oil, soybean oil, corn oil, sunflower oil, or peanut oil and esters, such as isopropyl myristate, can also be used as solvent systems for compound compositions. Additionally, various additives which enhance the stability, sterility, and isotonicity of the compositions, including antimicrobial preservatives, antioxidants, chelating agents, and buffers, can be added. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. In many cases, it will be desirable to include isotonic agents, for example, sugars, sodium chloride, and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monostearate and gelatin. According to the present invention, however, any vehicle, diluent, or additive used would have to be compatible with the compounds.

Sterile injectable solutions can be prepared by incorporating the compounds utilized in practicing the present invention in the required amount of the appropriate solvent with various of the other ingredients, as desired.

A pharmacological formulation of the present invention can be administered to the patient in an injectable formulation containing any compatible carrier, such as various vehicle, adjuvants, additives, and diluents; or the compounds utilized in the present invention can be administered parenterally to the patient

in the form of slow-release subcutaneous implants or targeted delivery systems such as monoclonal antibodies, vectored delivery, iontophoretic, polymer matrices, liposomes, and microspheres. Examples of delivery systems useful in the present invention include: 5,225,182; 5,169,383; 5,167,616; 4,959,217; 4,925,678; 4,487,603; 4,486,194; 4,447,233; 4,447,224; 4,439,196; and 4,475,196. Many other such implants, delivery systems, and modules are well-known to those skilled in the art.

A pharmacological formulation of the compound utilized in the present invention can be administered orally to the patient. Conventional methods such as administering the compounds in tablets, suspensions, solutions, emulsions, capsules, powders, syrups and the like are usable. Known techniques which deliver it orally or intravenously and retain the biological activity are preferred.

In one embodiment, the compound of the present invention can be administered initially by intravenous injection to bring blood levels to a suitable level. The patient's levels are then maintained by an oral dosage form, although other forms of administration, dependent upon the patient's condition and as indicated above, can be used. The quantity to be administered will vary for the patient being treated and will vary from about 100 ng/kg of body weight to 100 mg/kg of body weight per day and preferably will be from 10 μg/kg to 10 mg/kg per day.

The present invention also provides a method of diagnosing the presence of ischemia in a patient including the steps of analyzing a bodily fluid or tissue sample from the patient for the presence or gene product of at least one expressed gene (up-regulated) or their proteins and where ischemia is determined if the up-regulated gene or gene product is ascertained as described herein in the

Example. The bodily fluids can include tears, serum, urine, sweat or other bodily fluid where secreted proteins from the tissue that is undergoing an ischemic event can be localized. Additional methods for identification of the gene or gene product are immunoassays, such as and ELISA or radioimmunoassays (RIA), can be used as are known to those in the art particularly to identify gene products in the samples. Immunohistochemical staining of tissue samples is also utilized for identification. Available immunoassays are extensively described in the patent and scientific literature. See, for example, United States patents 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521. Further for identification of the gene, *in situ* hybridization, Southern blotting, single strand conformational polymorphism, restriction endonuclease fingerprinting (REF), PCR amplification and DNA-chip analysis using nucleic acid sequence of the present invention as primers can be used.

The above discussion provides a factual basis for the use of the sequences of the present invention to identify hypoxia-regulated genes and provide diagnostic probes to identify ischemia. The methods used with and the utility of the present invention can be shown by the non-limiting examples herein.

GENERAL METHODS

Most of the techniques used in molecular biology are widely practiced in the art, and most practitioners are familiar with the standard resource materials which describe specific conditions and procedures. However, for convenience, the following paragraphs can serve as a

guideline.

General methods in molecular biology: Standard molecular biology techniques known in the art and not specifically described were generally followed as in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1989), and in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989) particularly for the Northern Analysis and *In Situ* analysis and in Perbal, *A Practical Guide to Molecular Cloning*, John Wiley & Sons, New York (1988), and in Watson et al., *Recombinant DNA*, Scientific American Books, New York. Polymerase chain reaction (PCR) was carried out generally as in *PCR Protocols: A Guide To Methods And Applications*, Academic Press, San Diego, CA (1990).

Reactions and manipulations involving other nucleic acid techniques, unless stated otherwise, were performed as generally described in Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, and methodology as set forth in United States patents 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057 and incorporated herein by reference.

Additionally, *in situ* (In cell) PCR in combination with flow cytometry can be used for detection of cells containing specific DNA and mRNA sequences (Testoni et al, 1996, Blood 87:3822).

General methods in immunology: Standard methods in immunology known in the art and not specifically described are generally followed as in Stites et al.(eds), Basic and Clinical Immunology (8th Edition), Appleton & Lange, Norwalk, CT (1994) and Mishell and Shiigi (eds), Selected Methods in Cellular Immunology, W.H. Freeman and Co., New York (1980). Available immunoassays are

extensively described in the patent and scientific literature. See, for example, United States patents 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521 as well as Sambrook et al, *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor, New York, 1989.

GENERAL METHODS OF THE INVENTION

The general methods of the invention are generally as described in US patent application serial number USSN 09/309,862 of same applicant which is by reference incorporated herein in its entirety.

In brief, USSN 09/309,862 provides methods for identifying genes regulated at the RNA level by cue-induced gene expression. It relates to the rapid isolation of differentially expressed or developmentally regulated gene sequences through analysis of mRNAs obtained from specific cellular compartments and comparing the changes in the relative abundance of the mRNA in these compartments as a result of applying a cue to the tested biological samples. The cellular compartments include polysomal and nonpolysomal fractions, nuclear fractions, cytoplasmic fractions and splicesomal fractions. The method includes the steps of exposing cells or tissue to a cue or stimulus such as mechanical, chemical, toxic, pharmaceutical or other stress, hormones, physiological disorders or disease; fractionating the cells into compartments such as polysomes, nuclei, cytoplasm and splicesomes; extracting the mRNA from these fractions, and subjecting the mRNA to differential analysis using accepted methodologies, such as gene expression array

(GEM).

The method is designed for identifying and cloning genes which are either up- or down-regulated responsive to a specific pathology, stress, physiological condition, and so on, and in general, to any factor that can influence cells or organisms to alter their gene expression.

Further in USSN 09/309,862, an example is provided which shows the use of RNA isolation from nuclei for isolating genes whose steady state levels show only minor changes, but which show high differential expression when detected by nuclear RNA probe. Most such genes are regulated at the transcriptional level.

The specific mRNA of the invention is total cellular mRNA, and regulation is specifically on the transcriptional level.

DNA Microarrays

Preparation of custom hypoxia-specific microarrays

The cell system consisted of the C6 or A172 glioma cell lines. The cells were exposed to hypoxia for 4 or 16 hours and compared to cells grown under normal conditions.

Subtracted libraries were made from the following sample:

1. 16 hours hypoxia vs. normal (genes induced by hypoxia after 16 hours).
2. Normal vs. 16 hours hypoxia (genes reduced by hypoxia).
3. 4 hours hypoxia vs. normal (genes induced by hypoxia after four hours).

Three enriched libraries from the three groups above were made by the SSH method using the "PCR select

cDNA subtraction kit" from Clontech. From library 1, 1,000 colonies were grown and the plasmids prepared in 96 well format. From libraries 2 and 3, 500 colonies were processed from each. Thus, a total of 2,000 individual plasmids were prepared and used for the fabrication of a Gene Expression Microarray (GEM). For this, the inserts of each plasmid were amplified by PCR and robotically fabricated on the glass.

<u>Preparation of probes for microarray hybridization</u>

Isolated messenger RNA is labeled with fluorescent dNTP's using a reverse transcription reaction to generate a labeled cDNA probe. mRNA is extracted from either C6 or A172 cells cultured in normoxia conditions and labeled with Cy3-dCTP (Amersham) and mRNA extracted from C6 or A172 cells cultured under hypoxia conditions is labeled with Cy5-dCTP (Amersham). The two labeled cDNA probes are then mixed and hybridized onto microarrays (Schena et al, 1996). Following hybridization the microarray is scanned using a laser scanner and the amount of fluorescence of each of the fluorescence dyes is measured for each cDNA clone on the micro-array giving an indication of the level of mRNA in each of the original mRNA populations being tested. Comparison of the fluorescence on each cDNA clone on the micro-array between the two different fluorescent dyes is a measure for the differential expression of the indicated genes between the two experimental conditions.

The following probes were made from C6 and A172 for screening the GEM:

1. Normoxia (Cy3 labeled) + 16 hours hypoxia (Cy5 labeled).

2. Normoxia (Cy3 labeled) + 4 hours hypoxia (Cy5 labeled).

The following cDNA sequences were found to be induced under hypoxia conditions. The sequences are divided into three categories: 1. new genes; 2. known genes not known before this publication to be hypoxia-inducible; and 3. known genes known to be induced under hypoxia conditions.

In Situ Analysis:

In situ analysis is performed for the candidate genes identified by the differential response to exposure to hypoxia conditions as described above. The expression is studied in normal tissues and in pathological models as described herein.

Utilizing microarray hybridization the sequences set forth herein were identified and cloned as being differentially expressed under hypoxia conditions (see also Braren et al, 1997).

In parallel experiments Northern Analysis results and results obtained by the gene expression microarray analysis where found to coincide and either can be used to determine hypoxia-regulated response. As well in other experiments, the results from *in situ* analysis showed a high degree of correlation with the Northern Analysis and microarray analysis.

The sequences are listed that were found, the sequences are identified by clone number. In some cases either end of the clone has been sequenced for use or the entire clone sequence and protein sequence are provided.

Unigem1 (Syntheni) was utilized for screening of human glioma cell line A172 to identify genes whose expression is modified by hypoxia.

A Retinopathy Model:

Three major biological processes occur in nervous tissues under hypoxic conditions:

1. apoptotic death of hypoxia-damaged cells;
2. angiogenesis induced by factors secreted by hypoxia-suffering cells (a feedback control of oxygen concentration in tissue); and
3. secretion of neurotrophic and neuroprotective factors.

Therefore, it was assumed that among novel genes transcriptionally regulated by hypoxia in C6 and A172 glioma cells, there are those with pro- and antiapoptotic function as well as secreted neurotrophic, neuroprotective and angiogenic factors. It is worth noting, that regulation of apoptosis and angiogenesis is closely linked to cancerogenesis.

As initial step of biological characterization, candidate genes were tested for their ability to induce/protect cells from apoptosis, for neurotrophic activity and for angiogenic/antiangiogenic activity.

The sequences of the invention, the methods used therewith and the utility of the present invention can be shown by the following non-limiting examples:

EXAMPLE 1: 92 (SEQ ID. No.: 1)

Northern Blot Analysis:

Gene 92 is found upregulated after 16 hours of hypoxia. On Northern blots, it appears as a single 5 Kb transcript.

Cloning:

Several partial human cDNA clones corresponding to gene 92 were isolated from human A172 cDNA library. The length of available contig is 2212 bp and it contains an ORF potentially coding for a 437 amino acids (265-1576 bp) protein. The putative initiating ATG codon is preceded by in frame stop codon.

Bioinformatic Analysis:

Similarity search with 92 cDNA sequence against the public databases have shown 60% similarity to unknown Drosophila DNA sequence (AC004283) and mainly encompasses the 3' UTR and a part of the coding sequence. The search against the protein public databases gave partial similarity to hypothetical C. elegans protein (1703624) (77% similarity and 46% Identity).

The 92 cDNA sequence contains a region of 55 nucleotides (336-390 bp) that is constituted of CGG repeats. On the level of amino acids it appears as a GGD/SFGG repeated unit (aa 24-44). Two of the isolated cDNA clones contain a 30 nucleotides in frame deletion within this region, indicating that the amount of repeats can be variable. 44 of these nucleotides form a strong stem and loop secondary structure. When 92 cDNA was *in vitro* translated, the obtained protein had much smaller size than expected (30 kD instead of 45 kD). This, means that the stem and loop structure formed right downstream to the putative initiation codon prevents the proper progression of ribosome and the initiation actually starts from the next in frame ATG located at position 820 - 822.

EXAMPLE 2: 95 (SEQ ID. No.: 2)

Northern Blot Analysis:

Expression of this gene is upregulated by hypoxia. In normoxic conditions it appears as a single moderately expressed 3.9 Kb transcript. After four hours of hypoxia, an additional transcript of 4.3 Kb becomes evident. After 16 hours of hypoxia, an increase in the amount of the major 3.9 Kb transcript takes place and there appears the second additional transcript 4.5 Kb in size.

Cloning:

A complete human 95 cDNA clone, corresponding to the major 3.9 Kb mRNA was isolated. The contig is 3,535 bp long and it potentially codes for a 480 aa (ORF: nt 323-1763) protein. All the attempts to clone the gene 95 splice variants had thus far failed, although numerous different approaches were employed.

Bioinformatic And Literature Analysis:

Search against public databases revealed that gene 95 is similar to but not identical with a recently cloned human P53-responsive gene PA26 that codes for a nuclear protein (Oncogene, 18, 127 - 137, 1999). Like gene 95, the PA26 one is expressed in three splice variants. The major transcript is 2.6 Kb in size (T2) and is ubiquitously expressed, the second transcript is ~4 Kb (T1) and displays a certain tissue specificity of expression pattern. The third transcript is approximately 2.3 Kb (T3) and is very minor. Expression of 2.6 and 2.3 Kb transcripts is p53-regulated and is increased in response to irradiation, UV, doxorubicin etc. The 4 Kb transcript is not induced by p53. The PA26-specific transcripts share a set of C-terminal common exons, while the first exon is individual for each splice form. Gene 95

encoded putative protein shares the maximal amino acid homology with the common region of PA26-specific proteins. In its N-terminus, the putative protein 95 mostly resembles the T2 (major p53-inducible variant) variant of PA26.

PA26, maps to chromosome 6q21. Biologically it shares properties common to the GADD family of Growth Arrest and DNA Damage-inducible stress-response genes.

Domain Analysis:

A short sequence with features of second paroxysmal targeting signal was found in protein 95 between amino acids 353-361. A putative coiled coil region is found between amino acid positions 253-283.

Further Analysis Of Gene 95 Expression Pattern:

By analogy with gene PA26, it was assumed that alternative splicing in gene 95 also occurs in the first exons. To identify the mRNA species where the cDNA clone was derived from, two 95-specific probes were synthesized: (1) corresponding to the region homologous to the first exon of PA26 transcript T2 (Probe 1), and (2) corresponding to the region homologous to a common set of PA26 exons (but encompassing the sequences that are mostly diverged between the two genes) (Probe 2). On Northern blots, both probes recognized the same major 3.9 Kb 95-specific transcript that is up-regulated by hypoxia. No additional transcripts were identified by Probe 2. The 95-specific probe that was used for the initial set of hybridizations did not recognize any additional transcripts when hybridized to this newly prepared Northern blot. Therefore, the occurrence of 95-specific additional mRNA species seem to vary among different mRNA preparations.

The sizes of PA26 and gene 95 encoded transcripts are different. However, in order to exclude the possibility that the presence of alternative 95 RNA species stem from cross-hybridization, a PA26 specific cDNA probe was synthesized originating from the common exons region. On the Northern blot used for the previous experiment, the PA26 hybridized to two typical mRNA species of 2.6 and 4 Kb. Their levels, unlike the level of 95 mRNA, were not affected by hypoxia.

There is contradictory data in the literature about the nature of p53 protein present in A172 cells: according to some sources, it is wild type, according to others, mutated. The experiments performed thus far, conclude that hypoxia-induced expression of gene 95 is p53-independent.

The mammalian 95 expression vector was prepared and effects of this gene overexpression in mammalian cells were studied.

<u>Summary</u>:

95 is a novel hypoxia-induced human gene coding for a protein similar to p53-induced GADD protein PA26. However, the latter is inert to hypoxia stimulation. Therefore, there appears to exist two closely related proteins encoded by different genes that are upregulated by different genotoxic stresses. Protein 95 contributes to hypoxia-induced growth regulation in a way similar to PA26, but in a p53 independent manner. It is known that hypoxia-induced growth arrest that can take place in the core regions of p53-negative tumors is one of the obstacles for success of full chemotherapy. Therefore, inactivation of gene 95 can be a potent adjuvant for chemotherapeutic treatment of cancers.

EXAMPLE 3: 98 (SEQ. ID. No.: 3)

Northern Blot Analysis:

Expression of gene 98 is strongly up-regulated by hypoxia already after four hours of exposure. On Northern blots, it appears as a single mRNA species of 4.4. Kb.

Cloning:

A full-length 98 cDNA was cloned. It is 4138 bp long and contains an single ORF encompassing the nucleotides 204 - 1445. The putative protein is 414 amino acid a long.

Bioinformatic Analysis:

Search of the public databases revealed that 98 encoded protein is similar to two other human proteins: (1) a putative protein encoded by anonymous human 24945 mRNA sequence (AF131826) and (2) VDUP1 (protein induced in HL-60 cells by dihydroxy vitamin D3 treatment) (S73591). No significant structural features were found by existing protein analysis tools within the 98 putative protein.

It was previously demonstrated that treatment with vitamin D3 can induce apoptosis in C6 rat glioma cells (J. Neurosci Res,46, 540 - 550). Therefore, the relationship between the vitamin D-induced cell killing and 98 gene expression and function in glioma cells was studied.

The mammalian 98 expression vector was then and its effects studied.

EXAMPLE 4: 60F6 (SEQ. ID. No.: 4)

Northern Blot Analysis:

Expression of this gene is moderately up-regulated after 16 hours of hypoxia. On Northern blot, it appears as a single 3.0 Kb species.

Cloning:

A complete 60F6 human cDNA clone was isolated from A172 cDNA library. The contig is 2675 bp long and contains a single ORF (bp 134 - 866) able to code for a putative protein of 244 amino acids.

Bioinformatic Analysis:

A similarity search against the public databases revealed that the N-terminal half of 60F6 sequence exactly corresponds to a human cDNA coding for RhoE/Rho8 small GTP-binding protein (P52199, HSRHO8GRN). The identity of gene 60F6 was not determined before, because the small sequenced fragment that was initially possessed, originated from the Rho8 long 3' UTR. All the sequence information available in public databases did not include the long 3' UTR of Rho8. Structurally, Rho8 belongs to a family of Ras-related GTPases that regulate the actin cytoskeleton. However, this protein is unique in that it is constitutively active: GTPase deficient and *in vivo* farnesylated (Mol Cell Biol. 1996 Jun; 16(6): 2689-99). Therefore, it is intriguing to find that this constitutively active G-protein is regulated on the level of transcription. Hypoxia regulation of Rho8 was not previously described.

EXAMPLE 5: 648 (lysyl hydroxylase 2) (SEQ. ID. No.: 5)

Northern analysis

Probe 648 has detected a single 3.8 Kb

transcript on Northern blots. Expression was induced in C6 glioma cells already after 4 hours of hypoxia.

Cloning

After extension of initial cDNA probe by RACE it became evident that identified rat sequence is able to code for protein that represents a rat homologue of human lysyl hydroxylase 2 (PLOD2). The full- length open reading frames was cloned for both human and rat lysyl hydroxylase 2 homologues (by PCR, using primers built on the basis of known sequence, for human variant, and degenerative primers, for rat variant). The encoded proteins have well defined signal peptides.

Bioinformatics data

The cloned rat 648 cDNA contains an ORF coding for a putative protein that is 88% identical to the published human PLOD2 sequences. The least conserved sequences are within the signal peptide, however its functional features are completely preserved. The cloned human cDNA is almost identical to published human PLOD2 sequence. The word "almost" in the previous sentence stems from the fact that both in human and in rat cDNA species cloned in QBI a stretch of amino acids between positions 501- 521 of published sequence PLOD2 sequence was absent. Therefore, QBI's PLOD2 variants are differentially spliced. Both rat and human homologues were amplified from RNA extracted from glioma cell lines cultured in hypoxic conditions.

Literature review

Lysyl hydroxylases are the enzymes that catalyze the formation of hydroxylysine in collagens and other proteins with collagen-like amino-acid sequences, by the hydroxylation of lysine residue in X-K-G sequences. The

hydroxilysine residues have two important functions: (1) serve as sites of attachment of carbohydrate units, and (2) they are essential for the stability of the intermolecular collagen crosslinks. Congenital deficiency of lysyl hydroxylase in humans leads to increased solubility of collagens and, consequently, to numerous defects in organization of connective tissue in various organs. There are three known isoforms of lysyl hydroxilase, encoded by different genes. In humans, PLOD2 was found to be highly expressed in pancreas, skeletal muscle, heart and placenta (by Northern blot). Nothing is known either about the regulation of PLOD2 expression by hypoxia or about its involvement in angiogenesis and tumorigenesis. Induction of PLOD2 by hypoxia can probably account for hypoxia-induced tissue fibrosis. Indeed, specific lysyl hydroxylase inhibitor, minoxidil, was able to suppress both cellular collagen production and fibroblasts proliferation (J. Biol. Chem., 262, 11973 -8, 1987; Graefes Arch. Clin. Exp. Ophthalmol. 233, 347 -55, 1995). There were suggestions in literature to use modified lysyl hydroxylase inhibitor for treatment of vitreoretinopathy (Invest. Ophthalmol. Vis .Sci. 34, 567-75, 1993).

Analysis of alternatively spliced versions of gene 648

In order to establish whether the observed alternative splicing of PLOD2 is regulated by hypoxia, a set of PCR primers were synthesized that flank the alternatively spliced region. The expected sizes of RT-PCR products are: 216 bp, for published sequence and 156 bp, for the present sequence. Semi-quantitative RT-PCR was performed on RNA template extracted from human glioma A172 cells culture in either normoxia or in hypoxia for 4 and 16 hours. The obtained results clearly demonstrate that both PLOD2 forms are hypoxia regulated, but the form of

the invention appears only in hypoxic conditions.

<u>Testing potential pro- and antiapoptotic activity in transient transfection assays</u>

pcDNA3-648 was transiently co-transfected together with pcDNA3-GFP in Hela and 293 cells. 24 and 48 hours later the cells were fixed and stained with DAPI. No apoptotic effect was observed in the transfected cells. In order to evaluate the anti-apoptotic properties of the 648 protein, a co-transfection assay was conducted using the pcDNA3-GFP and the FAS plasmids. No anti-apoptotic effect was observed.

<u>Obtaining stable cell clones overexpressing 648 cDNA</u>

C6 were stably transfected with 5 ug of the pcDNA3-648 plasmid. Following G418 selection the level of expression was measured using Northern blot analysis in comparison to its level in C6 cells after 16 hours under hypoxic conditions. Out of 18 independent clones from the pcDNA3-648 transfection, no one was positive.

<u>*In situ* hybridization analysis</u>

<u>Retinopathy model</u>

Probe 648 demonstrates clear hybridization signal throughout the inner nuclear layer of "hypoxic" pup's retina while "normoxic" retina is negative for the expression. No hybridization signal was detected in adult retina.

In mouse embryo sections hybridization signal was detected in some apoptotic cells in the roof of the fourth brain ventricle and in developing retina ganglia, where expressing cells had no apoptotic features.

Multi-tissue block hybridization shows expression of 648 gene (rat PLOD2) in visceral smooth muscles in oviduct, uterus, stomach and intestine. Vascular smooth

muscles do not display hybridization signal.

Most prominent cell type hybridizing to 648 probe in ovary are granulosa cells of larger secondary follicles. No hybridization signal is detected in granulosa cells of primary and small secondary follicles. Significantly, hybridization signal is weakened in postovulatory follicles and completely disappears in corpora lutea. This shows that expression in granulosa cells is established at later stages of follicular maturation and it is abruptly down-regulated upon ovulation and the onset of conversion into lutein cells. On the other hand, follicular involution is not accompanied by the changes in 648 expression since strong hybridization signal is preserved in granulosa cells of atretic follicles.

Weak hybridization signal can be seen in some stromal cells surrounding large secondary follicles and corpora lutea as well as in cells of theca internal of secondary follicles. Prominent signal is found in "interstitial glands". This shows distinct regulation of 648 expression in theca cells undergoing "luteinization" in different locations: it is down regulated in corpora lutea but preserved or even upregulated in interstitial glands.

As to the germinal cells, an oocyte that expresses 648 was found only in one primary follicle while many other primary and secondary follicles had no hybridization signal. This shows a transient expression of 648 in oocytes at some stage of their development.

Discrepancy in the hybridization patterns of human (published) and rat PLOD2 (648) genes is explained by different sensitivities of different detection methods (Northern blot vs. *in situ* hybridization). The rat probe used in the present invention does not span an alternatively spliced region.

EXAMPLE 6: 24D4 (SEQ. ID. No.: 6)

Northern blot analysis

Expression of gene 24D4 is down-regulated after 16 hours of hypoxia. On Northern blots, it appears as a single 1.5 Kb mRNA species.

Cloning

A partial 24D4 human cDNA clone was isolated from A172 cDNA library. The available sequence is 1486 bp long and contains an N-terminal truncated ORF (bp 1-397).

Bioinformatic analysis

The sequence has no analogs in public databases. The available protein sequence contains three consequent Zn-finger motifs, all of C2H2 type (aa 52-72, 80-100 and 108-128). Zinc finger domains of this type are usually found in nucleic acid-binding proteins.

EXAMPLE 7: 77H4 (SEQ. ID. No.: 7)

Northern blot analysis

Expression of gene 77H4 is up-regulated after 16 hours of hypoxia. On Northern blots, it appears as a single mRNA species 0.6 -0.7 Kb in size.

Cloning

Several EST cDNA clones from public databases, corresponding to clone 77H4, were sequenced. All clones possess a poly A tail and a polyadenylation signal at their 3' end.

Bioinformatic analysis

EST clone 18E contains 580 bp, that include an ORF for a 120 aa protein (bp 92 – 452). The EST clone 3D contains 486 bp. Comparison of nucleotide sequences of clone 18E and 3D reveal that the latter has a out-of-frame deletion of 71 nucleotides between bp 279-339 of clone 18E (its putative coding region)(Fig.68). This raised the question whether 77H4 cDNA is at all coding. The sequence was analyzed by Genscan program, that predicts the potential coding sequences on the basis of codon usage. The only potentially coding segment was found in clone 18E between bp 93 and 235. An exhaustive search was performed of public databases for all 77H4-related sequences. Several independent contigs were identified in TIGR THC database. All of them are not completely identical to one another and contain nucleotide deletions of various length. This shows a certain variability in 77H4 nucleotide sequence.

Recently, a novel steroid receptor transcriptional coactivator, SRA, was found to be present as an RNA molecule in the transcription activating complex SRC-1 (Cell, 97, 17 - 27, 1999) . Although no similarity was found between clone 77H4 and SRA RNA on the sequence level, several characteristic features seem to be shared by both sequences:

•1 both mRNAs, 77H4 and SRA, are approximately of the same size - 0.7 Kb;

•2 sequencing multiple cDNA clones corresponding to either mRNA revealed extensive variability in certain regions;

•3 hybridization signals of both mRNA, therefore, appear as fuzzy bands on Northern blots;

•4 both mRNA do not exhibit characteristics of protein.

Therefore, the 77H4 cDNA clone has similar to

SRA function and can serve a coactivator in some transcriptional complexes.

EXAMPLE 8: 14G2 (SEQ. ID. No.: 8)

Northern blot analysis

Expression of gene 14G2 is regulated within 16 hours of hypoxia. On Northern blots, it appears as a single mRNA species.

Cloning

A partial 14G2 human cDNA clone was isolated. The available sequence was then characterized and cloned as shown in Figure 8.

EXAMPLE 9: 29F3 (SEQ. ID. No.: 9)

Northern blot analysis

Expression of gene 29F3 is regulated within 16 hours of hypoxia. On Northern blots, it appears as a single mRNA species.

Cloning

A partial 29F3 human cDNA clone was isolated. The available sequence was then characterized and cloned as shown in Figure 9.

Throughout this application, various publications, including United States patents, are referenced by author and year and patents by number. Full citations for the publications are listed below. The disclosures of these publications and patents in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the described invention, the invention can be practiced otherwise than as specifically described.

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Cell, 97, 17 - 27, 1999

CLAIMS

What Is Claimed Is:

1. A polynucleotide for use in identifying genes modulated by hypoxic conditions.

2. The polynucleotide according to claim 1, wherein said polynucleotide is selected from the group comprising nucleic acid sequence having SEQ. ID NOS. 1-9 and functional analogs thereof.

3. A diagnostic tool for identifying genes modulated by hypoxic conditions comprising detection means for detecting the presence of a polynucleotide having the acid sequence of SEQ. ID NOS. 1-9 and functional analogs thereof.

4. A pharmaceutical composition for modulating hypoxia and ischemia comprising an effective amount of a polynucleotide having the nucleic acid sequence according to SEQ. ID NOS. 1-9 and functional analogs thereof and a pharmaceutically acceptable carrier.

5. The pharmaceutical composition according to claim 4, wherein said pharmaceutical composition is used for gene therapy.

6. A pharmaceutical composition for modulating hypoxia and ischemia comprising an effective amount of a protein encoded by the nucleic acid sequence according to SEQ. ID NOS. 1-9 and functional analogs thereof and a pharmaceutically acceptable carrier.

7. A method of diagnosing the presence of a hypoxia associated pathology by screening for the presence at least one polynucleotide having the nucleic acid sequence according to one from the group comprising SEQ. ID NOS. 1-9 and functional analogs thereof.

8. A method of regulating hypoxic associated pathologies by administering an effective amount of a polynucleotide having a nucleic acid sequence from at least one from the group comprising SEQ. ID NOS. 1-9 and functional analogs thereof and a pharmaceutically acceptable carrier.

9. A method of regulating hypoxia associated pathologies by administering an effective amount of at least one antisense oligonucleotide against one of the nucleic acid sequences (SEQ. ID NOS. 1-9) or their proteins.

10. A method of regulating hypoxia associated pathologies by administering an effective amount of a protein encoded by the polynucleotide sequence from at least one of from the group comprising SEQ. ID NOS. 1-9 and functional analogs thereof as active ingredients in a pharmaceutically acceptable carrier.

11. A method of regulating hypoxia associated pathologies by administering an effective amount of a direct or indirect biologically active product of enzymatic activity of the protein encoded by the polynucleotide sequence from at least one of from the group comprising SEQ. ID NOS. 1-9 and functional analogs thereof as active ingredients in a pharmaceutically acceptable carrier.

12. A method of regulating hypoxia associated pathologies by administering an effective amount of inhibitors of a direct or indirect biologically active product of enzymatic activity of the protein encoded by the polynucleotide sequence from at least one of from the group comprising SEQ. ID NOS. 1-9 and functional analogs thereof as active ingredients in a pharmaceutically acceptable carrier.

13. A method of regulating hypoxia associated pathologies by inhibiting the enzymatic activity of the protein encoded by the polynucleotide sequence from at least one of from the group comprising (SEQ. ID NOS. 1-9) and functional analogs thereof as active ingredients in a pharmaceutically acceptable carrier.

14. A hypoxia response regulating gene.

15. The hypoxia response regulating gene according to claim 14, wherein said gene having the nucleic acid sequence according to SEQ. ID NOS. 1-9 and functional analogs thereof.

AMENDED CLAIMS

[received by the International Bureau on 15 February 2000 (15.02.00); original claims 1-12 replaced by new claims 1-12; original claims 13-15 cancelled (2 pages)]

1. A polynucleotide selected from the group consisting of SEQ ID Nos: 1-9, and functional analogs and complements thereof, which is differentially expressed and/or modulated under hypoxic conditions.

2. The polynucleotide according to claim 1 having hypoxia-regulating activity.

3. A diagnostic tool for identifying genes modulated by hypoxic conditions comprising detection means for detecting the presence of the polynucleotide of claim 1 and functional analogs thereof.

4. A method of diagnosing the presence of a hypoxia-associated pathology by screening for the presence of the polynucleotide of claim 1 and functional analogs thereof.

5. A method of regulating hypoxia-associated pathologies by administering a pharmaceutically effective amount of the polynucleotide of claim 1 and functional analogs thereof and a pharmaceutically acceptable carrier.

6. A method of regulating hypoxia-associated pathologies by administering a pharmaceutically effective amount of a protein encoded by the polynucleotide of claim 1 and functional analogs thereof as active ingredients in a pharmaceutically acceptable carrier.

7. A method of regulating hypoxia-associated pathologies by administering an effective amount of a direct or indirect biologically active product of enzymatic activity of a protein encoded by the polynucleotide sequence according to claim 1 and functional analogs thereof as active ingredients in a pharmaceutically acceptable carrier.

8. A method of regulating hypoxia-associated pathologies by administering a pharmaceutically effective amount of inhibitors of a direct or indirect biologically active product of enzymatic activity of a protein encoded by the polynucleotide sequence according to claim 1 and functional analogs thereof as active ingredients in a pharmaceutically acceptable carrier.

9. A method of regulating hypoxia-associated pathologies by inhibiting the enzymatic activity of a protein encoded by the polynucleotide sequence according to claim 1 and functional analogs thereof as active ingredients in a pharmaceutically acceptable carrier.

10. A pharmaceutical composition for modulating hypoxia and ischemia comprising an effective amount of a polynucleotide according to claim 1 and functional analogs thereof and a pharmaceutically acceptable carrier.

11. The pharmaceutical composition according to claim 10, wherein said pharmaceutical composition is used for gene therapy.

12. A pharmaceutical composition for modulating hypoxia and ischemia comprising a pharmaceutically effective amount of a protein encoded by the nucleic acid sequence according to SEQ ID NOS. 1-9 and functional analogs thereof and a pharmaceutically acceptable carrier.

GCACNAGGTGTGTGGCAGCAANAGCCGCCAGTTCGGGACCNCCGCANCTGGGGTGGCAAC
GGCGCAGGAGGGGTCGCGGGGAGGGAGTGGTGAGCGCAGGCGGCAGGGGTGTGGGAAAGA
CGAAGTCGCTATTTGCTGTCTGAGCGCGCTCGCAGCTCCTGGAAGTGTTGCCGCCTCTCG
GTTTCGCTCTCGCTCGCTGCGCTCCTAGAAGGGGCGGCCGCCTCCAGGACTGACCAGGGC
CAAGTGGCGCTCGGCGGGCACTACATGGCGGAGGGTGAAGGGTACTTCGCCATGTCTGAG
GACGAGCTGGCCTGCAGCCCCTACATCCCCCTAGGCGGCGACTTCGGCGGCGGCGACTTC
GGCGGCGGCGACTTCGGCGGTGGCGGCAGCTTCGGTGGGCATTGCTTGGACTATTGCGAA
AGCCCTACGGCGCACTGCAATGTGCTGAACTGGGAGCAAGTGCAGCGGCTGGACGGCATC
CTGAGCGAGACCATTCCGATTCACGGGCGCGGCAACTTCCCCACGCTCGAGCTGCAGCCC
AGCCTGATCGTGAAGGTGGTGCGGCGGCGCCTGGCCGAGAAGCGCATTGGCGTCCGCGAC
GTGCGCCTCAACGGCTCGGCAGCCAGCCATGTCCTGCACCAGGACAGCGGCCTGGGCTAC
AAGGACCTGGACCTCATCTTCTGCGCCGACCTGCGCGGGGAAGGGGAGTTTCAGACTGTG
AAGGACGTCGTGCTGGACTGCCTGTTGGACTTCTTACCCGAGGGGGTGAACAAAGAGAAG
ATCCACCAGTCACGCTCAAGGAAGCTTATGTGCAGAAAATGGTTAAAGTGTGCAATGAC
TCTGACCGATGGAGTCTTATATCCCTGTCAAACAACAGTGGCAAAAAATGTGGAACTGAAA
TTTGTGGATTCCCTCCGGAGGCAGTTTGAATTCAGTGTAGATTCTTTTCAAATCAAATTA
GACTCTCTTCTGCTCTTTTATGAATGTTCAGAGAACCCAATGACTGAGACATTTCACCCC
ACAATAATCGGGGAGAGCGTCTATGGCGATTTCCAGGAAGCCTTTGATCACCTTTGTAAC
AAGATCATTGCCACCAGGAACCCAGAGGAAATCCGAGGGGGAGGCCTGCTTAAGTACTGC
AACCTCTTGGTCAGGGGCTTTAGGCCCGCCTCTGATGAAATCAAGACCCTTCAAAGGTAT
ATGTGTTCCAGGTTTTTCATCGACTTCTCAGACATTGGAGAGCAGCAGAGAAAACTGGAG
TCCTATTTGCAGAACCTCTTTGTGGGATTGGAAGCCCGCAAGTATGAGTATCTCATGACC
CTTCATGGAGTGGTAAATGAGAGCTCAGTGTGCCTGATGGGACATGAAAGAAGACAGACT
TTAAACCTTATCACCATGCTGGCTATCCGGGTGTTAGCTGACCAAAATGTCATTCCTAAT
GTGGCTAATGTCACTTGATATTACCAGCCAGCCCCCTATGTAGCAGATGCCAACTTTAGC
AATTACTACATTGCACAGGTTCAGCCAGTATTCACGTGCCAGCAACAGACCTACTCCACT
TGGCTACCCTGCAATTAAGAATCATTTAAAAATGTCCTGTGGGGAAGCCATTTCAGACAA
GACAGGAGAGAAAAAAAAAAAAAAAAAAAAAAAAA

Fig. 1

CTCCGCGGCGGGGATGCTGAGGAGCGCTGGGTCCGGGAGCAGCCCTGGCCCCTGCGGACT
TCCGAGGCCGTGAAAACCCCTGCGCTGCGGCCCTTCCCAGGCCCCCTATTCCTTTCGCCG
TTCCCGAAGCCCGACTGGGGGAAGAGTCCAGCACCAAAGCGGCCGTTCTCGGATTCCGGA
GCGTTCTGGAGCCCCGAGAGACGCCCCGGGGTTCTAGAAGCTCCCCGGCGGCGCCCAGTC
CCGGCTTCATTCGGGCGTCCCTCCGAAACCCACTCGGGTGCACGGGTCGTCGGCGAGCCG
CGACCGGGTCCTGGCGCGCACCATGATCGTGGCGGACTCCGAGTGCCGCGCAGAGCTCAA
GGACTACCTGCGGTTCGCCCCGGGCGGCGTCGGCGACTCGGGCCCCGGAGAGGAGCAGAG
GGAGAGCCGGGCTCGGCGAGGCCCTCGAGGGCCCAGCGCCTTCATCCCCGTGGAGGAGGT
CCTTCGGGAGGGGGCTGAGAGCCTCGAGCAGCACCTGGGGCTGGAGGCACTGATGTCCTC
TGGGCGAGTAGACAACCTGGCAGTGGTGATGGGCCTGCACCCTGACTACTTTACCAGCTT
CTGGCNCCTGCACTACCTGCTGCTGCACACGGATGGTCCCTTGGCCAGCTCCTGGCGCCA
CTACATTGCCATCATGGCTGCCGCCCGCCATCAGTGTTCTTACCTGGTAGGCTCCCACAT
GGCCGAGTTTCTGCAGACTGGTGGTGACCCTGAGTGGCTGCTGGGCCTCCACCGGGCCCC
CGAGAAGCTGCGCAAACTCAGCGAGATCAACAAGTTGCTGGCGCATCGGCCATGGCTCAT
CACCAAGGAACACATCCAGGCCTTGCTGAAGACCGGCGAGCACACTTGGTCCCTGGCCGA
GCTCATTCAGGCTCTGGTCCTGCTCACCCACTGCCACTCGCTCTCCTCCTTCGTGTTTGG
CTGTGGCATCCTCCCTGAGGGGGATGCAGATGGCAGCCCTGCCCCCCAGGCACCTACACC
CCCGAGTGAACAGAGCAGCCCCCCAAGCAGGGACCCGTTGAACAACTCTGGGGGCTTTGA
GTCTGCCCGCGACGTGGAGGCGCTGATGGAGCGCATGCAGCAGCTGCAGGAGAGCCTGCT
GCGGGATGAGGGGACGTCCCAGGAGGAGATGGAGAGCCGCTTTGAGCTGGAGAAGTCAGA
GAGCCTGCTGGTGACCCCCTCAGCTGACATCCTGGAGCCCTCTCCACACCCAGACATGCT
GTGCTTTGTGGAAGACCCTACTTTCGGATATGAGGACTTCACTCGGAGAGGGGCTCAGGC
ACCCCCTACCTTCCGGGCCCAGGATTATACCTGGGAAGACCATGGCTACTCGCTGATCCA
GCGGCTTTACCCTGAGGGTGGGCAGCTGCTGGATGAGAAGTTCCAGGCAGCCTATAGCCT
CACCTACAATACCATCGCCATGCACAGTGGTGTGGACACCTCCGTGCTCCGCAGGGCCAT
CTGGAACTATATCCACTGCGTCTTTGGCATCAGATATGATGACTATGATTATGGGGAGGT
GAACCAGCTCCTGGAGCGGAACCTCAAGGTCTATATCAAGACAGTGGCCTGCTACCCAGA
GAAGACCACCCGAAGAATGTACAACCTCTTCTGGAGGCACTTCCGCCACTCAGAGAAGGT
CCACGTGAACTTGCTGCTCCTGGAGGCGCGCATGCAAGCCGCTCTGCTGTACGCCCTCCG
TGCCATCACCCGCTACATGACCTGACTCCTGAGCAGGACCTGGGCCCGGTTCAGCTCCCC
ACAAGGACTTCTCTGTCTGGAGACAGCCCCAGACCCTTTTGTGTCCCATGCCCACCCTCC
CCACGCTGCAGTGGGCTTGTGTGTGATGTGCAGTCCCGAAGCCACACCCTCCCTTTTCCT
CACTGGAATGGACAGTTCATTGCACTGACTCTGGGATCTCAGCCCTGCTCCTGGGAGCTG
GAAGAGCACTTGGAGATCCTAAGGGACCACACCCTTCCTCCTTCCCCTGCCCACAGAGGC
AGAGGGCACAGGAAAGAAGCCGGGCCAAGCTCGGAATTAATGTGCCACAAGTGTTGTGGC
CTTCCTGAACTGGGAAGTCCCTGGCTGGCCCCCGGGGGAGAGGGGCAAATGCCTCCGGGA
CTGACACTCCAGGCAGCTTTGCCTTCTCTCCCCTGTCATTTCCAGATTTCATTACCTCCT
ACTTGCCATTCACCCATCAATGTGAAAGTCAGGGTCACAGCTGGTCTGTGTGTCCAGTTC
CCTAAAAGCCTGTTCTGTTGGGCAGCCTGAGGCTGTTGCCCGAATCCTAGTTCAGTTTTT
TGACTTCCTTTGCCCTTTTTCCCTTTTCTCCATGCTTAATGGTGTGAGGCGTCAGGAGAG
AGGCCAAGTACATAAAAAAAAAAAAAAAGCAGATTATCTCTAGAGAGTTTGAGCCTTTGCT
GGTCACATTGCCTTCTGAAGAGGAGGGAGTATTAGATTATAAATCCTCTTTATTTTGGTC
CTTTATGCTTGAGGTTCCAACCTGGAGCCACAGTGTGTGANAGGAGGAGGAGAGGGAGAA
TTCTGTTCTCCCANAGCTGCACCTGCCTCGCANAGGCCAGCACCCCACTCTCCTGCCTCC
AGTGGCCCTGCCGCAGATGTCTCCCAAAAAGTTGAGCCTTTCTAGATGGCTTAGGTGGCA
CCATGGCTCAGCAGGAGGGGCGGGAGGCACCAGGGTTCTTGTTTGGACCCTGCCCCTGGG
CCATGGCCAGGTGACCATGGCTACATTGCCAAACCTCTGACTGCCACAGCTGCAGACTGA
GAGGGTGGGTCTGAGTCCCCACAATGTCTGAAGCTGCCCCTGGGATTCTCAGGCCAACCT

Fig. 2a

GCCAACAGCAAGCGGATTTTCTTGCAAGATCAGGGACCCCATTTCTGCAGCCAGTGTCTC
CTGGGTGCCTTCTGAGGACTCCCACCCCCATCCCAGTATCTCATCTGTCCCCTCTCCTGG
GGCTTAAGTGGGTTGCTTCCAGGCAGAAGCAGCCAAGGACCGATTCCAGGCACTTTCTGT
AGCAAATGACTGTGAATTACGACTTCTCTTGCCCTTCTTCTAGCAGTCTGTGCCTCCTCT
CTGACCAGTTTGGAGGGCACTGAAGAAAGGCAAGGGCCGTGCTGCTGCTGGGCGGGGCAG
GAGAGGAGCCTGGCCAGTGTGCCACATTAAATACCCGTGCAGGCGCGGAGAAGCAACCGG
CACCCCCTTCCGGCCTGAAAGCCCTCCCTGCAAGAAGGTGTGCAGGAGAGAAGAGGCCCC
GGCATGGGGATCTGGGTTCTAGAGGGCATGTGATGACTGTAAATGTTCACTGGGTGGGTA
GGGAGTGGTATCCAGTGTTCAAGTGCAGAAATCTTTGGCTTTGCTACCAGTTCCATATGA
TGAGAAATAAACGTTCGCTGAGGTTTTGTTTCATAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

Fig. 2b

GGCACGAGGACGAGCGGGAGCGCGGAGCAGCAGCCTCTGCTGCCCTGACTTTTTAAGAAA
TCTCAATGAACTATTTGTAGAGAATCACTGATCCGGCCTGCAAGCATTTTGCACGGCAAA
AATATCGATCAGTGTTAAGTGAAGATCACATTTTATATGCGATCTTGACTTTTTTGTCTT
ACATTATATTTTTATAGATTTTGTTATAAACATGGTGCTGGGAAAGGTGAAGAGTTTGAC
AATAAGCTTTGACTGTCTTAATGACAGCAATGTCCCTGTGTATTCTAGTGGGGATACCGT
CTCAGGAAGGGTAAATTTAGAAGTTACTGGGGAAATCAGAGTAAAATCTCTTAAAATTCA
TGCAAGAGGACATGCGAAAGTACGCTGGACTGAATCTAGAAACGCCGGCTCCAATACTGC
CTATACACAGAATTACACTGAAGAAGTAGAGTATTTCAACCATAAAGACATCTTAATTGG
GCACGAAAGAGATGATGATAATTCCGAAGAAGGCTTCCACACTATTCATTCAGGAAGGCA
TGAATATGCATTCAGCTTCGAGCTTCCACAGACACCACTCGCTACCTCATTCGAAGGCCG
ACATGGCAGTGTGCGCTATTGGGTGAAAGCCGAATTGCACAGGCCTTGGCTACTACCAGT
AAAATTAAAGAAGGAATTTACAGTCTTTGAGCATATAGATATCAACACTCCTTCATTACT
GTCACCCCAAGCAGGCACAAAAGAAAAGACACTCTGTTGCTGGTTCTGTACCTCAGGCCC
AATATCCTTAAGTGCCAAAATTGAAAGGAAGGGCTATACCCCAGGTGAATCAATTCAGAT
ATTTGCTGAGATTGAGAACTGCTCTTCCCGAATGGTGGTGCCAAAGGCAGCCATTTACCA
AACACAGGCCTTCTATGCCAAAGGGAAAATGAAGGAAGTAAAACAGCTTGTGGCTAACTT
GCGTGGGGAATCCTTATCATCTGGAAAGACAGAGACGTGGAATGGCAAGTTGCTGAAAAT
TCCACCAGTTTCTCCCTCTATCCTCGACTGTAGTATAATCCGCGTGGAATATTCACTAAT
GGTATATGTGGATATTCCTGGAGCTATGGATTTATTTCTTAATTTGCCACTTGTCATCGG
TACCATTCCTCTACATCCATTTGGTAGCAGAACCTCAAGTGTAAGCAGTCAGTGTAGCAT
GAATATGAACTGGCTCAGTTTATCACTTCCTGAAAGACCTGAAGCACCACCCAGCTATGC
AGAAGTGGTAACAGAGGAACAAAGGCGGAACAATCTTGCACCAGTGAGTGCTTGTGATGA
CTTTGAGAGAGCCCTTCAAGGACCACTGTTTGCATATATCCAGGAGTTTCGATTCTTGCC
TCCACCTCTTTATTCAGAGATTGATCCAAATCCTGATCAGTCAGCAGATGATAGACCATC
CTGCCCCTCTCGTTGAAGGAACACTTGGTTGAATCAAGTTGATGTGGGTTCCGAACTGTA
TCTCTTCCGGCTGAGGACAGAGAAGTATCTTGGAGACACGTTTCAGAGGAAGTGGAATTA
CTTTTGCCCAGAAAAATGGCGAATACATGAAACAACCAGTGATCATGCTTTAGAAGCCTA
CAGCAACATTCTGAGACTGCTCCAACATGCTTGAAGATCTAAGCTTTTCTCTTTTAAAAC
TGGCACATACTCAGAGCAGTCTTCTTAGCCTATGGTCGTACGTGTCAAGACATCACGTTG
TAAAGAGGGATGATTTCCTTCTTTTGATTTGAAAATTTGCACATGCTCAATGCTTACATT
GTGCGGTTCGACGTCACTACAGCTTCTTTTTTTTTTTTTTTTTTTCTATTTTTGCCAGAC
TCTTGATACTCTTAAAACTTGTTTGTGGTCAGCACAACAAGGAACAAAACAAAGCTTTGA
AAAAACTTTAACATGAAAAAAACGCACTGACATTTTTTTTATTTAATATAGCCTGGACTT
TACCTGCGTATGCACATGCTCAGAATTGTCTACTAGGCTGACTATGTATCACCTCTTCAG
CTTGGATCCAATTGTGGATTTATTTACAAACATCAAATGCCTTCAAGCCAATCCTTTTTG
CTGTATGTTTTGCAGCCTACTGTAGTAGATACGCAACAGATAATGTGGGAAAAAAAGAGA
TAAGAGGAGGAAGCTAATAAGAGACTGTCAAGATTGTATACCTTCTTGGTTTCTTTTAAG
AATTTGTTGCCTTTCTACTATTACAGCAAAGCAGCATTTTGTTACTGACTGCCTAAAATC
ACTTAATCTCAGGTGAACGCATCACTTGCCAAACTGTTGGAATGCTATTTGTGTTTTGTT
GCACTGTTTTTTTCGTTTGTTTGTTTGTTTATTTGGTTGGCTTTTTGGAGAGGGAAATTT
GGAAACGGGACATACACAAAAGTTACACACCCACATTCCCTTTTTATCATGACATACAAG
AAGAAACTAGCAGAGCTAAGAATGGAGTGAAGAAAGGCAGTATGGCAGGCACCAGCAAAG
AGTTGAGGGCTGTTGCTCTTAAAAATTATTTTTTTTATTATTATTTTGAAAGTATGGAAG
TTTTCCATTCACTGGGGAAAGGAGGGAAAAGTGCATTTATTTTTATACAGAGTTACTTAA
TTACCTCCAAAACACATATGTTGGAAATCGCTTTTGCTGGTGCAAAGTATATTAATGAGC
AGGAATACATACATTGAGGTTATGAATAGAGAGCTCAATTTGTACCTTTGCTGTCTTGCT
CAAGCTTGGTATGGCATGAAAACTCGACTTTATTCCAAAAGTAACTTCAAAATTTAAAAT
ACTAGAACGTTTGCTGCGATAAATCTTTTGGATTTTTGTGTTTTTCTAATGAGAATACTG
TTTTTCATTACCTAAAGAACAATTTGCTAAACATGAGAAATCACTCACTTTGATTATGTA

Fig. 3a

TAGATTACATAGGAAGAACAATCACATCAGTAAGTTATAGTTTATATTAAAGGTAATTTT
CTGTTGGCTCATAACAAATATACCAGCATTCATGATAGCATTTCAGCATTTTCCAAGGTA
CCAAGTGTACTTATTTTGTTGTTGTTGTTGTTGTTGTATTTTAGAAGGAATTCAGCTCTG
ATGTTTTTAAAGAAAACCAGCATCTCTGATGTTGCAACATACGTGTAAAATGGGTGTTAC
ATCTATCCTGCCATTTAACCCCACAGTTAATAAAGTGGCTGAAAATAATAGTAGCTCTGG
CTTGGTGCTTGACCTGGTTAAATACTGTCTTAAAGCTCATACAAAACAAATAGGCTTTTC
CATAAGTGGCCTTTAAGAAAACATGGAAGACAATTCATGTTTGACAAATGCTGACAGGGT
GAAGAAAGCCCAGTGTAAAAATGAATCGCGTTTTAAGTGATTCGGKTAAAGAGTTTGGGC
TCCCGTAGCAAACTAATACTAGATAATAAGGAAATGGGGGTGAAATATTTTTTTATTGTT
GAATCATTTTGTGAATGTCCCCCTCAAAAAAAGCTAATGGAATATTTGGCATAAAGGGCA
TTTGGTGGTTTTATTTTTGTTTGAGGGGGATTGTCAGAAAATCCCTTTTCTCTCTTACGT
CTAACTGACTAGGGAACAATTGTTGATATGCATAGCATTGGAATACTTGTCATTATATAC
TCTTACAAATAACACATGAAGCAAGAATGACCAATATTCTGATAATTGGCACTGGATCAC
AAAATGTGATAAAACTTTAAATGTATAAAACTTTATCAAATAAAGTTTTATTTTCCCCTT
TAAAATGTATTTCTTTAGAGGCATTACTTTTTTAAAAATATTGGTCAATTCCTGACATAA
GATGTGAGGTTCACAGTTGTATTCCAGTATTCAAGATAGATTCCTGATTTTTCAATTAGG
AAAAGTAAAATCCAAAATGTTAGCAAAACAAAGTGCAATATTAAATGTTTGCTTTATAGA
TTATATTCTATGGCTGTTTGTAATTTCTCTTTTTTTCCTTTTTTATTTGGTGCTGAATAT
GTCCTTGTAGGCTCTGTTTTAAGAAAACAATATGTGGGAAATGATTTAATTTTTCCTATT
GCTCTTCCTTGTGGAAAATAAAGTGTTTTGTTTTTTTCTGTTTTGTAAAAAAAAAAAAAA
AAAAAA

Fig. 3b

CCTCCTCCCCTCCTCCCGCCCSCGCCCCTGCTCCCCGCCCCCGGSSGCCCCNGCGGAACG
GTTACGCCGCGACGAAGTAAGGGTGGGTTCTNAAGGAAAGCCCTTTGCCAATCTTGCAAG
ATTTGTAGACCAGCACTACAAAGATCGCATAGATCAAATAGGAAAAAAAATGTCGATTTT
TATTCAGTCTGATGGTTCTGTTCTTCATTGTGATTGTCATTAAAAAGTGGTAAATTGCTC
AATGTAATATTTTTGTGCGCTGTTTAGAAGTTGTGTGATTTTTTGCCATCGTTGATAAAA
ATGCAAAGTCAAATAAAAGGTGTCTTGGTTTGATGTCATAGAATGATCCAAGGAGAGAAA
AAAGGTAGTTACTGTTTTCACCAGAAAAGGTAATGAGTGAAGGAAAGAATAGTAGCAGAA
AGCACAGTTTGTGAGTAAAGCTGTCTGGAATTAAGTTACCAAAAATACAAAGCAAAAGGA
CTATTATTTTGGGTTGAAGCTCCAAAACTGACAGCATCTGATAATCTGTTGGTTTATTTC
ACTTTTCATTAAATGAACATTGATGAGAGAAGATGCCACTTACCCAAGCTTTAGAGAATC
CCTAGTGGAAGATTATATGATAAACTTTCAGTCCTGACATAACACTAGGGCATTTCTAGA
GTGTCATTGCTAAAACCTCACTGAACAGACGCAGCCAAGGTCTGTGTTCAGCACTTGGTC
TCTGTTGTTACGTAAAATAATAAGCATTTAAAATAGTTTACAGATATTTTTGACCAGTTC
CTTTTAGAGATTCTTTCAGAGAAGAAACCAGATCTGACCTGTTTATTGTTGGCGCTTGTT
GAAAACGAGCTTTCTTTCCCATGATAGTGCTTCGTTTTTGAAGTGTTGAAGCTGTGCTCC
CCTTAAATCGTGGCAGGAGAGATTAAGGTAATTACAACACTCAGTTCTATGTCTTACAAG
CACTTTGTCTTGTCTCTGCAAGAAAATTCGATTCCAGTCATTTCCCATAAAATACAGACA
TTTTACCAACATAATATGCTTTGATTGATGCAGCATTATGCTTTGGGCAGTATTACAAAA
TAGCTGGCGAGTGCTTTCTGTATTTAAATATTGTAAAAATAAAATAAGTTATAACTGTTA
TAAAGCAGAACTTTTGTTGCATTTTTTAAACTGTTGAAGTCNCTGTGTATGTTTGTTTGG
TCAATGTTTCCNCAGTATTTATTAAAACATACTTTTTTTTTTCTTCAAATAAAAAAGTAA
CCATGTCTTTGTCTAAAAAAAAAAAAAANANNANNNNAAAAAAA

Fig. 4

ATGGGGGGATGCACGGTGAAGCCTGAGCTGCTGCTCCTGGCGCTCGTCCTCCACCCCTGG
AATCCCTGTCTGGGTGCGGACTCGGAGAAGCCCTCGAGCATCCCCACAGATAAATTATTA
GTCATAACTGTAGCAACAAAAGAAAGTGATGGATTCCATCGATTTATGCAGTCAGCCAAA
TATTTCAATTATACTGTGAAGGTCCTTGGTCAAGGAGAAGAATGGAGAGGTGGTGATGGA
ATTAATAGTATTGGAGGGGGCCAGAAAGTGAGATTAATGAAAGAAGTCATGGAACACTAT
GCTGATCAAGATGATCTGGTTGTCATGTTTACTGAATGCTTTGATGTCATATTTGCTGGT
GGTCCAGAAGAACTTCTAAAAAAATTCCAAAAGGCAAACCACAAAGTGGTCTTTGCAGCA
GATGGAATTTTGTGGCCAGATAAAAGACTAGCAGACAAGTATCCTGTTGTGCACATTGGG
AAACGCTATCTGAATTCAGGAGGATTTATTGGCTATGCTCCATATGTCAACCGTATAGTT
CAACAATGGAATCTCCAGGATAATGATGATGATCAGCTCTTTTACACTAAAGTTTACATT
GATCCACTGAAAAGGGAAGCTATTAACATCACATTGGATCACAAATGCAAAATTTTCCAG
ACCTTAAATGGAGCTGTAGATGAAGTTGTTTTAAAATTTGAAAATGGCAAAGCCAGAGCT
AAGAATACATTTTATGAAACATTACCAGTGGCAATTAATGGAAATGGACCCACCAAGATT
CTCCTGAATTATTTTGGAAACTATGTACCCAATTCATGGACACAGGATAATGGCTGCACT
CTTTGTGAATTCGATACAGTCGACTTGTCTGCAGTAGATGTCCATCCAAACGTATCAATA
GGTGTTTTTATTGAGCAACCAACCCCTTTTCTACCTCGGTTTCTGGACATATTGTTGACA
CTGGATTACCCAAAAGAAGCACTTAAACTTTTTATTCATAACAAAGAAGTTTATCATGAA
AAGGACATCAAGGTATTTTTTGATAAAGCTAAGCATGAAATCAAAACTATAAAAATAGTA
GGACCAGAAGAAAATCTAAGTCAAGCGGAAGCCAGAAACATGGGAATGGACTTTTGCCGT
CAGGATGAAAAGTGTGATTATTACTTTAGTGTGGATGCAGATGTTGTTTTGACAAATCCA
AGGACTTTAAAAATTTTGATTGAACAAAACAGAAAGATCATTGCTCCTCTTGTAACTCGT
CATGGAAAGCTGTGGTCCAATTTCTGGGGAGCATTGAGTCCTGATGGATACTATGCACGA
TCTGAAGATTATGTGGATATTGTTCAAGGGAATAGAGTAGGAGTATGGAATGTCCCATAT
ATGGCTAATGTGTACTTAATTAAAGGAAAGACACTCCGATCAGAGATGAATGAAAGGAAC
TATTTTGTTCGTGATAAACTGGATCCTGATATGGCTCTTTGCCGAAATGCTAGAGAAATG
ACTTTAVAAAGGGAAAAAGACTCCCCTACTCCGGAAACATTCCAAATGCTCAGCCCCCCA
AAGGGTGTATTTATGTACATTTCTAATAGACATGAATTTGGAAGGCTATTATCCACTGCT
AATTACAATACTTCCCATTATAACAATGACCTCTGGCAGATTTTTGAAAATCCTGTGGAC
TGGAAGGAAAAGTATATAAACCGTGATTATTCAAAGATTTTCACTGAAAATATAGTTGAA
CAGCCCTGTCCAGATGTCTTTTGGTTCCCCATATTTTCTGAAAAAGCCTGTGATGAATTG
GTAGAAGAAATGGAACATTACGGCAAATGGTCTGGGGGAAAACATCATGATAGCCGTATA
TCTGGTGGTTATGAAAATGTCCCAACTGATGATATCCACATGAAGCAAGTTGATCTGGAG
AATGTATGGCTTCATTTTATCCGGGAGTTCATTGCACCAGTTACACTGAAGGTCTTTGCA
GGCTATTATACGAAGGGATTTGCACTACTGAATTTTGTAGTAAAATACTCCCCTGAACGA
CAGCGTTCTCTTCGTCCTCATCATGATGCTTCTACATTTACCATAAACATTGCACTTAAT
AACGTGGGAGAAGACTTTCAGGGAGGTGGTTGCAAATTTCTAAGGTACAATTGCTCTATT
GAGTCACCACGAAAAGGCTGGAGCTTCATGCATCCTGGGAGACTCACACATTTGCATGAA
GGACTTCCTGTTAAAAATGGAACAAGATACATTGCAGTGTCATTTATAGATCCCTAAGTT
ATTTACTTTTCATTGAATTGAAATTTATTTTGGATGAATGACTGGCATGAACACGTCTTT
GAAGTTGTGGCTGAGAAGATGAGAGGAATATTTAAATAACATCAACAGAACAACTTCACT
TTGGGCCAAACATTTGAAAAACTTTTTATAAAAAATTGTTTGATATTTCTTAATGTCTGC
TCTGAGCCTTAAAACACAG

Fig. 5a

ATGGGGGGATGCACGGTGAAGCCTCAGCTGCTGCTCCTGGCGCTCGTCCTCCAGGGGTGG
AATCCCTGTCTGGGTGCGGACTCGGAGAAGCCCTCGAGCATCCCCACAGATAAATTATTA
GTCATAACTGTAGCAACAAAAGAAAGTGATGGATTCCATCGATTTATGCAGTCAGCCAAA
TATTTCAATTATACTGTGAAGGTCCTTGGTCAAGGAGAAGAATGGAGAGGTGGTGATGGA
ATTAATAGTATTGGAGGGGGCCAGAAAGTGAGATTAATGAAAGAAGTCATGGAACACTAT
GCTGATCAAGATGATCTGGTTGTCATGTTTACTGAATGCTTTGATGTCATATTTGCTGGT
GGTCCAGAAGAAGTTCTAAAAAAATTCCAAAAGGCAAACCACAAAGTGGTCTTTGCAGCA
GATGGAATTTTGTGGCCAGATAAAAGACTAGCAGACAAGTATCCTGTTGTGCACATTGGG
AAACGCTATCTGAATTCAGGAGGATTTATTGGCTATGCTCCATATGTCAACCGTATAGTT
CAACAATGGAATCTCCAGGATAATGATGATGATCAGCTCTTTTACACTAAAGTTTACATT
GATCCACTGAAAAGGGAAGCTATTAACATCACATTGGATCACAAATGCAAAATTTTCCAG
ACCTTAAATGGAGCTGTAGATGAAGTTGTTTTAAAATTTGAAAATGGCAAAGCCAGAGCT
AAGAATACATTTTATGAAACATTACCAGTGGCAATTAATGGAAATGGACCCACCAAGATT
CTCCTGAATTATTTTGGAAACTATGTACCCAATTCATGGACACAGGATAATGGCTGCACT
CTTTGTGAATTCGATACAGTCGACTTGTCTGCAGTAGATGTCCATCCAAACGTATCAATA
GGTGTTTTTATTGAGCAACCAACCCCTTTTCTACCTCGGTTTCTGGACATATTGTTGACA
CTGGATTACCCAAAAGAAGCACTTAAACTTTTTATTCATAACAAAGAAGTTTATCATGAA
AAGGACATCAAGGTATTTTTTGATAAAGCTAAGCATGAAATCAAAACTATAAAAATAGTA
GGACCAGAAGAAAATCTAAGTCAAGCGGAAGCCAGAAACATGGGAATGGACTTTTGCCGT
CAGGATGAAAAGTGTGATTATTACTTTAGTGTGGATGCAGATGTTGTTTTGACAAATCCA
AGGACTTTAAAAATTTTGATTGAACAAAACAGAAAGATCATTGCTCCTCTTGTAACTCGT
CATGGAAAGCTGTGGTCCAATTTCTGGGGAGCATTGAGTCCTGATGGATACTATGCACGA
TCTGAAGATTATGTGGATATTGTTCAAGGGAATAGAGTAGGAGTATGGAATGTCCCATAT
ATGGCTAATGTGTACTTAATTAAAGGAAAGACACTCCGATCAGAGATGAATGAAAGGAAC
TATTTTGTTCGTGATAAACTGGATCCTGATATGGCTCTTTGCCGAAATGCTAGAGAAATG
GGTGTATTTATGTACATTTCTAATAGACATGAATTTGGAAGGCTATTATCCACTGCTAAT
TACAATACTTCCCATTATAACAATGACCTCTGGCAGATTTTTGAAAATCCTGTGGACTGG
AAGGAAAAGTATATAAACCGTGATTATTCAAAGATTTTCACTGAAAATATAGTTGAACAG
CCCTGTCCAGATGTCTTTTGGTTCCCCATATTTTCTGAAAAAGCCTGTGATGAATTGGTA
GAAGAAATGGAACATTACGGCAAATGGTCTGGGGGAAAACATCATGATAGCCGTATATCT
GGTGGTTATGAAAATGTCCCAACTGATGATATCCACATGAAGCAAGTTGATCTGGAGAAT
GTATGGCTTGATTTTATCCGGGAGTTCATTGCACCAGTTACACTGAAGGTCTTTGCAGGC
TATTATACGAAGGGATTTGCACTACTGAATTTTGTAGTAAAATACTCCCCTGAACGACAG
CGTTCTCTTCGTCCTCATCATGATGCTTCTACATTTACCATAAACATTGCACTTAATAAC
GTGGGAGAAGACTTTCAGGGAGGTGGTTGCAAATTTCTAAGGTACAATTGCTCTATTGAG
TCACCACGAAAAGGCTGGAGCTTCATGCATCCTGGGAGACTCACACATTTGCATGAAGGA
CTTCCTGTTAAAAATGGAACAAGATACATTGCAGTGTCATTTATAGATCCCTAAGTTATT
TACTTTTCATTGAATTGAAATTTATTTTGGGTGAATGACTGGCATGAACACGTCTTTGAA
GTTGTGGCTGAGAAGATGAGAGGAATATTTAAATAACATCAACAGAACAACTTCACTTTG
GGCCAAACATTTGAAAAACTTTTTATAAAAAATTGTTTGATATTTCTTAATGTCTGCTCT
GAGCCTTAAAACACAGATTGAAGAAGAAAAGAAAGAAAAAACTTAAATATTTATTTCTAT
GCTTTGTTGCCTCTGAGAATAATGACAATTTATGAATTTGTGTTTCAAATTGATAAAATA
TTTAGGTACAAATAACAAGACTAATAATATTTTCTTATTTAAAAAAAGCATGGGAAGATT
TTTATTTSTCSSSSTSTSGSGGSSSTGTSGSCSSSSTGGSTSTSSSTGSSSSTTSCCSTG
TTGTAAAACCTTGAAAATCAGATTCTAACTGATTGTATGCAACTAAGTATTTCTGAACAC
CTATGCAGGTCTTATTTACAGTGTTACTAAGGGAACACACAAAGAATTACACAACGTTTT
CCTCAAGAAAATGGTACAAAACACAACCGAGGAGCGTATACAGTTGAAAACATTTTTGTT
TTGATTGGAAGGCAGATTATTTTATATTAGTATTAAAAATCAAACCCTATGTTTCTTTCA
GATGAATCTTCCAAAGTGGATTATATTAAGCAGGTATTAGATTTAGAAAACCTTTCCATT
TCTTAAAGTATTATCAAGTGTCAAGATCAGCAAGTGTCCTTAAGTCAAATAGGTTTTTTT
TTGTTGGTGGTTGTGCTTGCTTTCCTTTTTTAGAAAGTTCTAGAAAATAGGAAAACGAAA
AATTTCATTGAGATGAGTAGTGCATTTAATTATTTTTTAAAAAACTTTTTAAGTACTTGA

Fig. 5b-a

ATTTTATATCAGGAAAACAAAGTTGTTGAGCCTTGCTTCTTCCGTTTTGCCCTTTGTCTC
GCTCCTTATTCTTTTTTGGGGGGAGGGTTATTTGCTTTTTTATCTTCCTGGCATAATTTC
CATTTTATTCTTCTGAGTGTCTATGTTAACTTCCCTCTATCCCGCTTATAAAAAAATTCT
CCAACAAAAATACTTGTTGACTTGATGTTTTATCACTTCTCTAAGTAAGGTTGAAATATC
CTTATTGTAGCTACTGTTTTTAATGTAAAGGTTAAACTTGAAAAGAAATTCTTAATCACG
GTGCCAAAATTCATTTTCTAACACCATGTGTTAGAAAATTATAAAAAATAAAATAATTTT
AAAAAAAAAAAAAAAAAAAAAAA

Fig. 5b-b

GGCTGCGAGAAGACGACAGAAGGGGTCCGTCGTCTGCTCGGTGCGCTCGGGCTCCGCGCT
AGTCCGCTCAGTGTTCTCCAATCGCTTTGGTACCCACGCAGTCCTCTCATCCGTCCTCCG
CTGCCGTCCCGGGCCCCACGTCTAACCCGGTGCTCTTCGGGGTCTCCGCGTCTCGCGAGA
AGTCCTCGCCGCAGGCCTCGGGCTTTCGGGCTTAGGGGCGGATGGGGGACCGCGGAGTGA
GGCTGGGGCTGCTGATGCCCATGCTCGCCCTGCTCTCCTGGGCGGCTAGCCTGGGCGTAG
CGGAGGAGACTCCCTCGCGCATCCCAGCAGATAAGTTATTAGTCATAACTGTAGCAACCA
AAGAAAACGATGGATTCCACAGATTTATGAATTCAGCCAAGTATTTCAATTATACTGTGA
AGGTTCTTGGTCAAGGGCAAGAGTGGAGAGGTGGTGATGGGATGAACAGTATTGGAGGGG
GCCAGAAGGTGAGATTAATGAAAGAAGCCATGGAGCACTACGCCGGTCAGGACGATCTGG
TCATCTTGTTTACTGAATGTTTTGATGTTATATTTGCTGGTGGGCCTGAAGAACTTCTTA
AAAAGTTCCAAAAGACAAATCATAAAATCGTCTTTGCAGCGGATGCGCTGTTGTGGCCAG
ATAAGCGGCTGGCAGACAAGTATCCTGGTGTGCACATTGGGAAACGCTACCTGAATTCTG
GAGGCTTTATTGGCTATGCTCCCTACATCAGCCGTCTGGTCCAGCAGTGGGATCTGCAGG
ATAATGATGACGACCAGCTCTTTTACACTAAAGTTTACATCGACCCGCTGAAAAGGGAAG
CTCTTAACATCACATTGGATCACAGATGCAAAATTTTCCAGGCCTTGAATGGAGCTACAG
ACGAAGTTGTTTTAAAGTTTGAAAATGGTAAAAGCAGAGTGAAGAATACATTTTATGAAA
CACTGCCAGTGGCCATCAATGGGAATGGGCCCACCAAAATTCTCTTGAATTACTTTGGAA
ACTATGTTCCAAATTCATGGACACAGGAAAATGGCTGTGCTCTTTGTGACTTTGACACAA
TTGACCTGTCTACAGTAGATGTCTATCCGAAGGTAACACTAGGTGTTTTTATTGAACAAC
CAACCCCCTTTCTACCTCGGTTCCTGGACTTACTGTTAACACTGGATTACCCTAAAGAAG
CACTTCGACTCTTTGTCCATAATAAAGAAGTTTATCATGAAAAGGACATCAAAGCGTTTG
TTGATAAAGCTAAACACGACATCAGCTCTATAAAAAATAGTAGGACCAGAGGAAAATCTAA
GTCAAGCGGAAGCCAGAAACATGGGAATGGATTTCTGCCGTCAGGATGAAAAGTGTGATT
ACTACTTTAGTGTGGATGCAGATGTTGTTTTGACAAACCCAAGAACTTTAAAAATTTTGA
TTGAACAAAACAGGAAGATCATTGCCCCTCTTGTGACACGTCATGGAAAGTTGTGGTCCA
ACTTCTGGGGAGCCCTGAGTCCTGATGGATACTATGCTCGTTCTGAAGATTACGTAGATA
TCGTTCAGGGAAACAGAGTAGGAATATGGAATGTCCCATACATGGCTAATGTGTACTTAA
TTCAAGGGAAGACGCTGCGATCAGAGATGAGTGAAAGGAACTATTTTGTGCGTGATAAGT
TGGATCCCGACATGTCTCTCTGCCGCAATGCTCGAGACATGACCTTACAAAGGGAAAAAG
ACTCCCCCACTCCGGAAACATTCCAAATGCTCAGCCCCCCAAAGGGTGTGTTTATGTACA
TTTCTAACAGACATGAATTTGGACGGCTGATATCAACTGCTAATTACAACACTTCCCATC
TCAACAATGACCTCTGGCAGATCTTTGAAAATCCCGTGGATTGGAAGGAAAAATATATAA
ACCGTGACTATTCAAAGATTTTCACTGAAAATATAGTCGAGCAGCCCTGTCCAGATGTCT
TCTGGTTTCCCATATTTTCTGAACGAGCCTGTGACGAGTTGGTAGAAGAAATGGAACATT
ACGGCAAGTGGTCCGGGGGAAAGCATCATGACAGCCGTATATCTGGTGGCTATGAAAATC
TCCCAACGGATGACATTCATATGAAGCAGATTGACCTGGAGAACGTCTGGCTTCACTTTA
TCCGAGAGTTTATCGCTCCAGTTACCCTGAAGGTCTTCGCGGGATATTACACCAAGGGAT
TTGCCCTGCTGAACTTCGTAGTGAAGTACTCGCCCGAAAGACAGCGCTCGCTCCGGCCTC
ACCACGATGCGTCAACCTTCACCATCAACATTGCTCTAAATAATGTAGGAGAGGATTTTC
AGGGAGGTGGATGCAAATTCCTAAGGTATAATTGCTCCATCGAATCCCCCCGAAAAGGCT

Fig. 5c-a

GGAGCTTCATGCATCCTGGGAGGCTTACTCATCTACACGAAGGGCTTCCTGTCAAAAATG
GAACAAGATACATTGCAGTCTCATTTATCGATCCCTAAGTTATTGACTGAACTTAAACTG
AGTGGCTCTTTGAGATGGATGACTGGCGGGAACATGTCTCTGAAGTTGTACTTGAGAAGA
CGAGAGGAATATTTAAATAATGTCACCAGAACAACGTCACTTTGGGCCAAGCATTTGAAA
ACTTTTTATATAAATTTGTTTTATGTTTCTTAACGTCTGCTCTGAGCCTTAAAACACAGG
TTGAAGAAGAAGAGAGAGGAAAAAAGTGAAAGTTGGTATTTATTTCTGTGCTTTAATTGT
CTATGAAAATGATGACATTTTATAAAATGTTTAGGTACAAAGGCATGAATGATAATCAGT
AAGCCTAATAATATTTTCTTATTTAAGGAGAACCTGAGAAGATTTTATTTTTCAGTGGGA
GAAATATGGAAAATGGTTCTAAATGAGGGTCGGCACGTCTGGAAGCCCGGGATTCTGACG
CGTACTGAATTTATGTGTAACTTTTAAGCCATGCTGACCTCCGGGTAGATTCGCTTTTCA
GTGATAAGGAAGAAAACCCAAAGAAAATATTGCACAGAGGCTTTCCTCAAGCAGCCTGGG
CAGATGGCCAGTGGAAGCCCATCCACTGGAGATCCTCAGCTTGTGAGGCAGGTGCTCCTG
TCCGTTGGAAACTGGGCCCCTGTGTGTCTCCAGGGCAAGCTCTCAGGGGAAGCTCACATC
TGCCTGCTTTACAGAGTGCTTCAGGCGTCAGCTCCAAGTCAAACAGGATGTGTTTCCTTC
TGTTTTTCCCCTCTAATTATAGAAAATAGTAAGGAAAAATATCAGTTTCATTGAGATTAG
TAGTACATTTTACTATCTTCTTTTTTAACGATTAAGTACTTGAATTTTATATCAGGAAAA
TAGTTTTTGAGCCTGTTCTTACCTTTGGCCGTAGTTGGTAGTTGGTCTCTTTGTTTTTCC
TGGAGGAGGGGCATTTCTTTTCCTCATCATAAACTACTTTCTCATTCTTAGTCTTGTTAT
TACTTTTCCTCTACCCCACTTTTTAAAAATTCCCACAGCAAAATTTTTATTTGAATTTTT
AATATTTCTCTGAATGAGGTTTAAATATCTTTATTAGAGCTACTGTTTTTAATTTAAAGG
TTAAACTTGAAGAAAGTCTTTATTCATGGTGCCAAAATGCATTTTTCTAACTCTGTGTGT
TAGAAAATAATGAAAAATAAAATAACTTACAATAAAAAAAAAAAAAAAAAAAAAAAA

Fig. 5c-b

CGGCCCTTCCCTTGCCACATCTGCGGCAAGGGCTTCATCACGCTCAGCAACCTCTCCAGG
CACCTGAAGCTGCACCGGGGCATGGACTGACTGCCAGGCTGCGTGCGCCCTGCCCTCCAC
CCAGCCTCCTGGACTCGGCCTGGACCAGGGGACCTCGGGACTGCGCGTGAGGCCCCGGCC
CTCCAAATCCAAATCCAGACGCAGGCCCTGAAATGAGGGGACCCTGACTGGAGAGGTGGG
GGCCACCAAAAACCCACAAAGGCCCCGGAGCTGGGGGACCACAAACAAACAGGGTCCTTA
GCTGGGGCAGGGGAGCCCAAATCTAGGGAGAGACTCCTGAGCCTGAGGTCCCTGGAATGA
GTGTGGGTAGCCGTAAGTCCCCAAGACATGGGGACTTTGCAGTGAGCAATGGGTCTCCAC
AAGTACCTCTCATCTTGAGAGCCCTAATACTAAAAGATGGGCACCCACCCCCACCAAGGG
AAGACTGCCCCATTCCCTGAGAGCCATCATTCCTAACGACCTTGATCTGGAGAATGTGGA
GGGAGCATGTCCCTGAATTTTCCTAGATCCCTCCAAATGCCACCCACCAGAGTCACTGGT
GACCCCAGAAAATGGATATAGCCGAAATCTGCCTTTCCCCTTTTTCATTCCCTGTGCTGA
AAGAGGGACCAGGGTAGATGCCCCCTGCCCTCGAATCCCCCCTCCCCGACTGTGGAATGG
ATCGACCCTAACGATCTTCCCCGCCCCAAACACTAGAATAGACTGGCCTGAAATCCCCTT
GCCCAGTAGAATGGACTGATCTATGTGCACACACCCCCATCACATGGAATGGGCTGGTCT
AGGCTGTGGCCTGCCACCTTCCTTAGAGTGAATAGGGGGGACACTCCTTTTTTTTTCCTG
TAGGGTGTGGGCCGGTCCACGCAATTTTTTATCCTGTGAACTCATTTGAGTGGGAGGTGG
TGGACACCTGGGGTTTCCTTNCCTNTTTCCGTAGCATCCGTTGGTNTTTTTTTCCATNTN
TGTTGGNTTGTNTGTTTNTGTTTTCCTCCCNATCCCTAGGGGAAGGGGGCNTTTGGNTAG
GGGGNGNCCCTGTNAGCCTNGNCCTTGCCCCCTTGTNCCTTTNCCCAGTGTTTNCAGGNC
CCCCNATNAACCTTGTCCTGTCAGTNGNTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAA

Fig. 6

TTTCTTTTTTTCTTTCGGCGTCTGCGGTGCTCGGAAGTGTGGTACTTCTCCTAGTTGCAG
TCAGGCTTCATACGCTATTGTCCTGCCCGTTAGAGCAGCCAGCGGGTACAGAATGGATTT
TGGAAGAGGGAGTCACCACTGGACCTCCAAGGAAGCCACGTGCAGACATCTACAACCTTC
GATCTCCTGACGAGTTTATTGTTGGCCAAAACCAGGCTTTGATTGAACCAGGATGAATGC
GGGTGTTGGAAGTAGAATATATATATACATATAAAATTGGTTGGGAGCCACGTGTACCAG
TGTGTGTTGATCTTGGCTTGATTCAGTCTGCCTTGTAACAGAAACTGGCGATGGAATATG
AGAGGAGCCCTCTGGAAAGAAAAGGACAGACCCTGTGCTTTCATGAAAGTGAAGATCTGG
CTGAACCAGTTCCACAAGGTTACTGTATACATAGCCTGAGTTTAAAAGGCTGTGCCCACT
TCAAGAATGTCATTGTTAGACTTTGAAATTTCTAACTGCCTACCTGCATAAAGAAAATAA
AATCTTTTAAATCAAAAAAAAAAAAAAAAAAAAAAAAAAA

Fig. 7a

GGCACGAGGGAGTGTGGTACTTCTCCTAGTTGCAGTCAGGCTTTATACGCTGTTGTCCTG
CCCGTTAGAGCAGCCAGCGGGTACAGAATGGATTTTGGAAGAGGGAGTCACCACTGGACC
TCCAAGGAAGCCACGTGCAGACATCTACAACCTTCGATCTCCTGACGAGTTTATTGTTGG
CCAAAACCAGGCTTTGATTGAACCAGGATGAATGCGGGTGTTGGAAGTAGAATATATATA
TACATATAAAATTGAAACTGGCGATGGAATATGAGAGGAGCCCTCTGGAAAGAAAAGGAC
AGACCCTGTGCTTTCATGAAAGTGAAGATCTGGCTGAACCAGTTCCACAAGGTTACTGTA
TACATAGCCTGAGTTTAAAAGGCTGTGCCCACTTCAAGAATGTCATTGTTAGACTTTGAA
ATTTCTAACTGCCTACCTGCATAAAGAAAATAAAATCTTTTAAATCAAAAAAAAAAAAAA
AAAAAA

Fig. 7b

GGCACGAGAGGCACAGCACGACCTCTATGCAGACAAGTGAACTGTAGAAACTGATTACTG
CTCCACCAAGAAGCCCCCATAAGAGTGGTTATCCTGGACACAGAAGTGTTGAATTGAAAT
CCACAGAGCATTTTACAAGAGTTCTGACCTGGATGGGGTAAACCTCAGTGCACTTCTTTT
CTGTTGGCCTCAGTATTACTGGATTGAAGAATTGCTGCTTCTTGTTAGGAGGTTCATTTC
ACTTATCATTACTTACAACTTCATACTCAAAGCACTGAGAATTTCAAGTGGAGTATATTG
AAGTAGACTTCAGTTTCTTTGCATCATTTCTGTATTCAATTTTTTTAATTATTTCATAAC
CCTATTGAGTGTTTTTTAACTAAATTAACATGGCTCGAATGAACCGCCCAGCTCCTGTGG
AAGTCACATACAAGAACATGAGATTTCTTATTACACACAATCCAACCAATGCGACCTTAA
ACAAATTTATAGAGGAACTTAAGAAGTATGGAGTTACCACAATAGTAAGAGTATGTGAAG
CAACTTATGACACTACTCTTGTGGAGAAAGAAGGTATCCATGTTCTTGATTGGCCTTTTG
ATGATGGTGCACCACCATCCAACCAGATTGTTGATGACTGGGTAAGTCTTGTGAAAATTA
AGTTTCGTGAAGAACCTGGTTGTTGTATTGCTGTTCATTGCGTTGCAGGCCTTGGGAGAG
CTCCAGTACTTGTTGCCCTAGCATTAATTGAAGGTGGAATGAAATACGAAGATGCAGTAC
AATTCATAAGACAAAAGCGGCGTGGAGCTTTTAACAGCAAGCAACTTCTGTATTTGGAGA
AGTATCGTCCTAAAATGCGGCTGCGTTTCAAAGATTCCAACGGTCATAGAAACAACTGTT
GCATTCAATAAAATTGGGGTGCCTAATGCTACTGGAAGTGGAACTTGAGATAGGGCCTAA
TTTGTTATACATATTAGCCAACATGTTGGCTTAGTAAGTCTAATGAAGCTTCCATAGGAG
TATTGAAAGGCAGTTTTACCAGGCCTCAAGCTAGACAGATTTGGCAACCTCTGTATTTGG
GTTACAGTCAACCTATTTGGATACTTGGCAAAAGATTCTTGCTGTCAGCATATAAAATGT
GCTTGTCATTTGTATCAATTGACCTTTCCCCAAATCATGCAGTATTGAGTTATGACTTGT
TAAATCTATTCCCATGCCAGAATCTTATCAATACATAAGAAATTTAGGAAGATTAGGTGC
CAAAATACCCAGCACAATACTTGTATATTTTTAGTACCATACAGAAGTAAAATCCCAGGA
ACTATGAACACTAGACCTTATGTGGTTTATTCCTTCAATCATTTCAAACATTGAAAGTAG
GGCCTACATGGTTATTTGCCTGCTCACTTTATGTTTACATCTCCCACATTCATACCAATA
TACGTCAGGTTTGCTTAACCATTGATTTTTTTTTTTTTTTACCAAGTCTTACAGTGATTAT
TTTACGTGTTTCCATGTATCTCACTTTGTGCTGTATTAAAAAAACCTCCATTTTGAAAAT
CTACGTTGTACAGAAGCACATGTCTTTAATGTCTTCAGACAAAAAAGCCTTACATTAATT
TAATGTTTGCACTCTGAGGTGCAACTTAACAGGGAGGGCCTGAGAAAAGAATGGGAGGGG
GCTATTAATTATTTTTAGCAAAATGTTGCCTTTGTCTTGTGCAAACATGTAGAATATGCT
CTTTAATTTAGTAAAATATTTTTTTAAAAGGTAGAGATGCTTTGGTATTGGTATCATAAA
CTTCCTGAAATTCTTGAATTTTTTTCCCATACTATCAAGAAGTGTGTTTACCACTTATTT
TTGTTTGAAAGTGTGATTTTTTTTTTCCTTCCCAACCTCTCCTTGCAAAAAAAGAAATGG
GTTTCTGCTAATGAATTGAGCAGACATCTAATATTTTATATGCCTTTTGAGCTGTGTAAC
TTAATATTTGGATACTTGACAATTTGTTTTATTATGTAATTGATAAAATGGTGATGTGTA
TTAATGTTAGTTCAACCATATATTTATACTGTCTGGGGATGTGTGGTTATAGTTCTGTGG
GAGAAATAATTTTGTCAGTGTTCACCAGCTTGTAAAAACTTAGTGCGAGAGCTGAAACAT
CTAAATAAATAATGACATGCATTTATCATCATTGAGATTGGTTTGCTTAAAATTAACTTA
TTTTGTAGAAGACAAAATGAATTGCACTTCACTTAATGTGTGTCCTCATCTTTTTACAAA
TAAATGAAGGATTATAAATGATGTCAGCATTTTAGTAAACTTTTAGACAAAATTTGTTAG
GGTCATTCATGAAAACTTTAATACTAAAAGCACTTTCCATTATATACTTTTTAAAGGTCT
AGATAATTTTGAACCAATTTATTATTGTGTACTGAGGAGAAATAATGTATAGTAGAGGAC
AGCCTTGGTTTGTAAAGCTCAGTTCCACTAGTTCATGGTTTTGTGCAACTTTTGAGCCTC
AGTTTTCTCCTTTGCAAATTAATAATTACATACCTTTATAGATTTTGAAATTAATTTAAA
TATTAGTATTTGGACATGAAGGCTTAATGTTAAGTTTCCTTTAATGATCCACAATAATCC
CTTTGATCACGTTAATCTAAATCTAGATGTCTTTGTCTAATTTTTTTTGAATAGCAGTTA
TAAATGTAAAGGACTCAAAGTTTAAGTAAAAAGTGATACTCCACCTTGTGTTTCAAAGAA
TTTAGTTCCACCTCTTCATACCAGTTTAACACTTAATATATTTCATTGGATTTTAGACAG
GGCAAAAGGAAGAACAGGGGCCTCTGGAGGCCCTTGGTTATTTAAATCTTGGATTATTTG
TGATAGTAATCACAAATTTTTGGCTAATTTTTAACCTGAGGTTTTGTTTTTTTTTTTAAA
GGAAATGCAGCCTAGTCTTGAGAACATAATTTTATATAATCAATTACTAAATGTTAAACT
ATTACCACACAGCCCATAAAACAGCATTTGCGTTTATTGAGAGAGAGGATGTGCCATCAT

Fig. 8a

GATTAATGAAAACTATCTTTTGAGTTTGAAAAGAAATTAATTTGCAGTGTTTGGATTGTA
TATATGGTGCTAAAAATAAATTAATTTACTTTATAAACCTTATCTGTACATTATACGATG
TGATGAAATTTGCTTTTTATCCAAATATTTTGTATCTTGTAAATATGGCTAATTATAGGA
ATGCCTATAATACATCTTAGATTCCTTATATCTAATAAGAGTTCAAAGAGTTATGAGTTG
AAGTCTTGAATGCAGGAAACTATCTGATAGTGTTCTAAAATTTGGTTACTTGGGTTTGGA
TACCCTTAGTGGGATGATGTAAATAGAGGCTAGCTACCTAGGCTTGTCTATAGCAACCAT
AATGTTGATGTAAGTAATGCGGTTACTGAATCATAAGAAAATGCCATCTCTTTTTAGTTG
AAGGAAAACTCTGGAAGTAGGTGCCATTGGTCATTCTGCAGTGCACTGCAACCATTGTTT
CCCCTAGTGCCCTCTTTTCCCTAGGGCATTGCTCTCCTATTCCCACGCCTTAACACAGCT
CTATACCTAGAAGCAGCCAGCCCAGGCATGCAGTCACATTTAATCACATCCCCCTTCTAG
AGTGCTTCAAAATGATGTAGTCCCTCAACTTGGCTAAAGAATCTCAATCTCTTGAAATTT
ATTTTTTTAATGTCATATTCATCTGGTAAATATCTACTGTTTGCCAGGCATTTAAGAATA
TGGCAAAGAACATAAAAGATGGTGTCACCAGATTTTGGTCACCAATGAGTACCCGACCCG
TTGCCATGATTAAGAGAGAATGCTTTCTATTGGAGTTTCAGGAAATATAATTTGAGAATA
CTTTAAAGGGAAGTGGAAGTATAAGTGAATGATATTTTTCTTTTACATGTAAACAATGAA
GTTATTTCAAAGTTAAGTTTTAAACAAAATCCATGAAGTAGTGTCTGCCATACATGTTAA
TATTCTACATTCTTGCTTCCCTTAAATTAATATGTTTGTGTGTATATATGTGCCTCACAC
CTGAATTGAAAATTAAAGACTGGTTTAAAAGTGGTTTAAAAGTGACATTTAATGTTTCTC
CATTACGTTTGGGGTAACCAGCCTAAGTGGAATCTTGGAAGGAAAGTAAGGGAAAAACTT
GTATTTGCCTTCAATGAATTAAACCAGTGATATGTTTAACGTATGAATGAAAGGATTGAT
GGTGATTTTATAATTATATATATTGCCGCAGTAACCAGTTAATAAATTGATAGCTACCAT
TTAAAAAAAAAAAAAAAAAAAA

Fig. 8b

GCCAAGAATTCGGCACGAGGAGTGGGTATCTGAGTTAGTCAGTTTTACCATTTATAAAAT
GTTTGGTGGAGGAAATTGAAACTAATTGCTAAATTGTTAGTAACCAGTGCATTAACTAGG
ACCCTACTGAGTGGACTGAAAGAATCGAAAATGTTTAACTGGTTGAGAGGCAATGATGTT
GCAAATGGGGTATTCTTCAAAGCTCCTTCTGGGGGGAAATCTTCAAAGGCAATTATTCTG
AATGTAAACTACAGACCAAATTGCAGTCTTCTGTAAGCATTTCAGAGATTSCCTCAAATA
TTTTTTGATTAAAAAACTCTTCCGTGGTCTTTTGTGCTTCAGAACTACCCAGTACAACAG
GGTCTTCAGCCTGCTCAGGATCTCTAAAGAGAGCTAGCACACAGTCAGCCAACTTTGGCT
GCTTCAACTCCTAGGAACAAGAAATGATGCTGAGATAATTTGTCTGGCAGGTATTATCAG
CCCACAATGACTGCTGTCATTTAGCCTCAAAATGTTTATTTTTTTTTTTACAATGCTGTA
TTTCTTTAGAACCTTCCTATTCCGAGTGTGGACCCTAGGCCAGCCCCATAGACTTCCCCT
GGGGACTTGTCAGAAATGCATAATTTTAGGCCCCACCCCAGACCTGTTGGACCAGAATCT
TCATTTAACAAGATGCCCAGGTGATTCATTCATGTTTGAGAAGCTCTGCTTTAAATCACT
AAAGCAGTTACTGAGTAATTACTACCATCATGACTCTGAAGAGCTCCTATAGCCTTCAAA
TGCACCTAACTCTACTCTAAAGGCAAATGTCCTCACTGGGAAATCTGATCTGCTGTTTCA
GAGAAGTGCAGGGCTACACAGTGTCTTACACTCCTATCTATTGATGTTTCTTGGTTTTGC
CTGGTAATCTGCTGCTTAAATGGATTATTTGATGACATATTGATATTAAAACAGTCCTAT
TTTTAGAAAAAAAAAAAAAAAAAAA

Fig. 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/20394

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/00
US CL :536/23.1, 23.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1, 23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS
search terms: hypoxi###, regulat####, respons####, gene, DNA, polynucleotide, nucleic acid, cDNA, mRNA, RNA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,731,294 A (ROBINSON et al.) 24 March 1998, column 2, line 53 to column 3, line 10; column 5, lines 33-56.	1, 14

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
09 NOVEMBER 1999	08 DEC 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SCOTT D. PRIEBE Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/20394

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 2-13, 15
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

   Claims 2-13 and 15 are directed to SEQ ID NOs: 1-9. However, no 'Sequence Listing' has been filed either in paper copy or computer readable form as required under PCT Rule 5.2. Consequently, no meaningful search can be performed on these claims.

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

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